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(54) Title: SCREENING ASSAYS FOR THE DETECTION AND DIAGNOSIS OF INFLUENZA VIRUS

(57) Abstract

The present invention encompasses rapid, specific assay systems for detecting and diagnosing influenza virus infections by assessing for the presence of influenza virus neuraminidase. The present invention also encompasses a rapid, specific, high through put assay system for identifying novel agents that modulate influenza virus neuraminidase activity. The present invention further encompasses a rapid, specific, high through put assay system for identifying novel agents that interact with influenza virus neuraminidase.

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SCREENING ASSAYS FOR THE DETECTION AND DIAGNOSIS OF INFLUENZA VIRUS

1. FIELD OF THE INVENTION

The present invention relates to screening assays and kits, and methods of employing them for the detection and diagnosis of influenza virus infection. In particular, the present invention relates to screening assays for the detection and diagnosis of influenza virus infections based on rapid, specific assay systems for detecting influenza neuraminidase. The present invention further encompasses kits for the diagnosis of influenza viral infection based on the species specific detection of influenza neuraminidase. Furthermore, the present invention relates to rapid, specific, high through-put assay systems to screen for agents that interact with influenza virus neuraminidase and which may have utility as antiviral agents.

2. BACKGROUND OF THE INVENTION

2.1 INFLUENZA VIRUS INFECTION

Influenza virus infection is an important clinical problem worldwide. Influenza has been known for centuries to occur in recurrent epidemics that initiate abruptly, spread rapidly, and are frequently worldwide. Indeed, influenza was responsible for one of the most devastating plagues in history; between 1917 and 1918 approximately 20 million people were killed as a result of influenza infections. Although epidemics occur periodically, outbreaks of influenza occur annually. In the United States alone, up to 40 million people develop influenza infections each year. Of those individuals, approximately 150,000 are hospitalized, and 10,000 to 40,000 die from the flu or flu-related complications (Welch, S., 1988, *Gilead's Oral Influenza Drug Proves Positive in Phase III*, BioWorld Today 9:1,3). In the United States approximately $10 billion is spent annually for doctor's visits, lost productivity, and wages as a result of influenza virus infections.

Influenza is an acute respiratory disease associated with constitutional symptoms. The disease results from the destruction of cells lining the upper respiratory tract, the trachea, and the bronchi due to influenza virus infection. Influenza virus enters the nasopharynx and spreads to cells which express specific mucoprotein receptors. Although the virus must pass through respiratory secretions, which contain mucoproteins that the viral particles can combine with, infection is not blocked because the viral neuraminidase hydrolyzes the mucoproteins, rendering them ineffective as inhibitors.

Acute infection with influenza virus results in viral replication, which is followed by necrosis of infected cells and extensive desquamation of the respiratory epithelium. This is directly responsible for the respiratory symptoms associated with acute infection. The constitutional symptoms associated with acute influenza virus infection include fever, chills, generalized aching, headache, prostration, and anorexia. Normally, the disease resulting from influenza virus infection is self-limited and lasts 3 to 7 days. Secondary bacterial infections (e.g., *Staphylococcus aureus*, *Hemophilus influenzae*, and β-hemolytic streptococci) account for most deaths from influenza. Rarely, does infection with influenza alone result in death.

2.2 INFLUENZA VIRUS

Influenza viruses are enveloped viruses containing a negative-sense segmented single-stranded RNA genome. Influenza viruses are classified as members of the Orthomyxoviridae family. On the basis of their nucleocapsid and M protein antigens, influenza viruses have been further classified into three types (genus), influenza A, B, and C. New variants of influenza A and B types are continually emerging and are classified into subtypes (species) based upon the expression of immunologically distinct surface antigens, the hemagglutinin (HA) and the neuraminidase (NA)

glycoproteins. The antigenic variations of these two surface antigens is due to antigenic drift. There are two distinct forms of antigenic drift: minor antigenic drift and major antigenic drift. Minor antigenic drift reflects changes due to mutations in the HA and NA genes of the virus. Major antigenic drift results from recombination (i.e., gene reassortment) between human and animal strains of influenza virus. Thus, viruses can reassort genes during mixed infections and produce new virus species against which a large proportion of the world population is immunologically defenseless.

The influenza virions consist of an internal ribonucleoprotein core (a helical nucleocapsid) containing the single-stranded RNA genome, and an outer lipoprotein envelope lined inside by a matrix protein (M). The segmented genome of influenza A and B consists of eight molecules (seven for influenza C) of linear, negative polarity, single-stranded RNAs which encode ten polypeptides, including: the RNA-directed RNA polymerase proteins (PB2, PB1 and PA) and nucleoprotein (NP) which form the nucleocapsid; the matrix proteins (M1, M2); nuclear export protein (NEP) two surface glycoproteins which project from the lipoprotein envelope: hemagglutinin (HA) and neuraminidase (NA); and a nonstructural proteins (NS1). A summary of the genes of the influenza virus and their protein products is shown in Table I below.

TABLE I

INFLUENZA VIRUS GENOME RNA SEGMENTS AND CODING ASSIGNMENTS[a]

Segment	Length[b] (Nucleotides)	Encoded Polypeptide[c]	Length[d] (Amino Acids)	Molecules Per Virion	Comments
1	2341	PB2	759	30-60	RNA transcriptase component; host cell RNA cap binding
2	2341	PB1	757	30-60	RNA transcriptase component; initiation of transcription; endonuclease activity?
3	2233	PA	716	30-60	RNA transcriptase component; elongation of mRNA chains?
4	1778	HA	566	500	Hemagglutinin; trimer; envelope glycoprotein; mediates attachment to cells
5	1565	NP	498	1000	Nucleoprotein; associated with RNA; structural component of RNA transcriptase
6	1413	NA	454	100	Neuraminidase; tetramer; envelope glycoprotein
7	1027	M_1	252	3000	Matrix protein; lines inside of envelope
		M_2	96		Structural protein in plasma membrane; spliced mRNA
8	890	NS_1	230		Nonstructural protein
		NEP	121		Nuclear Export Protein

[a] Adapted from R.A. Lamb and P. W. Choppin (1983), Reproduced from the Annual Review of Biochemistry, Volume 52, 467-506.
[b] For A/PR/8/34 strain
[c] Determined by biochemical and genetic approaches
[d] Determined by nucleotide sequence analysis and protein sequencing

Hemagglutinin (HA) and neuraminidase (NA) are two major surface glycoproteins expressed by influenza viruses. HA mediates attachment of the virion to the host cell, the first step of viral infection, by binding to terminal sialic acid residues in glycoconguates. In contrast to HA activity, NA catalyzes removal of terminal sialic acids linked to glycoproteins and glycolipids. The role of NA in the infectious process is unclear. It has been postulated that NA activity is required for the release of newly formed

viruses from infected cells by digesting sialic acids in the HA receptor. Furthermore, NA may promote viral movement through respiratory tract mucus, thereby enhancing viral infectivity.

For type A influenza strains, NA has been classified into nine subtypes based on their serological properties. Type B influenza virus does not have any subtypes. The NA of influenza virus types A and B only share 30% amino acid sequence homology (Kim, C.H. et al., 1997, Journal of the American Chemical Society 119:681). However, the enzyme activity of NA among the different strains is the same, indicating the highly conserved nature of the active site of the enzyme. NA molecules form tetrameric spikes consisting of a slender stalk topped by a box-like head region. The X-ray crystallographic structures of NA has been determined for three influenza subtypes: A/Tokyo, A/Tern, and B/Beijing (Varghese, J.H. and Coleman, P.M., 1991, Journal of Molecular Biology 221:473; Bossart-Whitaker, P. et al., 1993, Journal of Molecular Biology 232:1069; Burmeister, W.P. et al., 1992, EMBO Journal 11:49). The structures indicated that NA consists of a symmetrical folding pattern of six four-stranded anti-parallel β-sheets arranged like blades of a propeller. The crystallographic studies have revealed that the amino acids which line and surround the walls of the binding pocket in the active site are highly conserved among all examined influenza virus strains.

Although much work has been focused on the discovery of inhibitors of influenza virus neuraminidase (von Itzstein, M. et al., 1993, Nature 363:418-423; Kim, C.H. et al., 1997, Journal of the American Chemical Society 119:681-690; Bischofberger, N. et al., United States Patent Number 5,763,483; Kim, C. et al., United States Patent Number 5,512,596; Kim, C. et al., International Patent Application Number WO 98/17647; Bischofberger, N. et al., International Patent Number WO 96/26933; Colman, P. et al., International Patent Application Number WO 92/06691; Luo, M. et al., United States Patent Number 5,453,533), there is still not an

effective inhibitor of neuraminidase available for the treatment of influenza. In fact, there is currently no effective drug for the treatment of influenza. Presently, the only available means for treating influenza viral infection is prophylactically through vaccination. However, developing an effective vaccine requires correctly predicting the strain of influenza that will be endemic the next "flu season." Thus, there still exists a need for a drug which will effectively inhibit influenza viral infection.

2.3 CURRENT METHODS AVAILABLE FOR DIAGNOSIS OF INFLUENZA VIRUS INFECTION

A variety of methods are available for the clinical diagnosis of influenza virus infection. Traditionally, influenza virus has been detected by inoculating cell cultures with biological samples and assessing the presence of virus using hemagglutinin inhibition, ELISA, or immunofluorescence assays. Although this method is highly sensitive and specific, the time required for culture, isolation, and identification can range from 2 to 10 days. Since influenza virus infection is normally self-limited, this method is not useful for diagnosis.

Influenza virus infection can be detected and diagnosed by immunologic methods, which detect the presence of viral specific antibodies or viral specific antigens. A variety of immunologic techniques are available for detecting viral specific antibodies and viral specific antigens, including ELISAs (enzyme linked immunosorbent assays), solid-state radioimmunoassays, and immunofluorescent assays. The clinical diagnosis of influenza virus infection based upon the detection of viral specific antibodies requires that an increase in antibody titer is demonstrated since most individuals already have antibodies against influenza viruses at the time of infection. On the other hand, the detection of viral specific antigens utilizing immunologic methods depends on the use of antibodies that recognize an influenza

virus antigen and consequently, new strains of virus may not be detected. Furthermore, immunologic methods for detecting influenza virus require a laboratory and someone with technical expertise to perform the assays.

Influenza virus infection can also be detected and diagnosed based upon the enzymatic activity of neuraminidase. Various assays utilizing these approaches have been described in the literature (e.g., Santer, U.V. et al., 1978, Biochimica et Biophysica 523:435-442; Potier, M. et al., 1979, Analytical Biochemistry 94:287-296; Yolken, R.H. et al., 1980, Journal of Infectious Diseases 142:516-523; von Itzstein, M. et al., 1993, Nature 363:418-423; Turner, G. et al., International Patent Application Number WO 91/09975; Turner, G. et al., International Patent Application Number WO 91/09972; Turner, G. et al., International Patent Application Number WO 91/10744; Turner, G. et al., International Patent Application Number WO 91/09971; Reece, P. A. et al., International Patent Application Number WO 97/32214; Liav, P.A. et al., United States Patent Number 5,719,020). However, the utilization of the above-referenced neuraminidase enzymatic activity dependent assays for the diagnosis of influenza is questionable due to their lack of sensitivity and/or specificity. Colorimetric and fluorometric detection systems are not sensitive enough to detect the low concentrations of neuraminidase found in some biological samples. Although fluorometric detection systems are more sensitive than colorimetric detection systems, the fluorescence of biological materials is affected by high protein levels, which quench the fluorescent signal. Furthermore, a variety of organisms contain neuraminidase including mammals, bacteria (*Vibro Cholerae*, *Clostridium perfringens*, *Streptococcus pneumoniae*, and *Arthrobacter sialophilus*) and viruses (parainfluenza virus, mumps virus, Newcastle disease virus, fowl plaque virus and sendai virus) and the currently available neuraminidase assays are not sensitive enough to distinguish between these viruses.

Clinical diagnosis by laboratory tests are generally too costly for individual or sporadic cases. Additionally, the tests utilized to diagnose influenza virus infection are time consuming and require a laboratory in order to perform them. Furthermore, there has been no reliable treatment available to individuals suffering from influenza infection. Thus, individuals suffering from influenza virus have had to rely on the presumptive diagnosis made by physicians with little reliable treatment available. Therefore, a need exists for a simple, rapid, and accurate diagnostic kit for influenza virus infection which can be performed in a physician's office.

3. SUMMARY OF THE INVENTION

The present invention relates to assays that can be used for the detection and diagnosis of influenza virus infection, and for the identification of agents that have anti-influenza viral activity. The assays of the invention utilize influenza viral neuraminidase (NA) as the target, and are based, in part, on the Applicants' design of highly sensitive and specific assay systems for the detection of influenza virus NA, ligands or compounds that bind specifically to influenza virus NA, and/or ligands or compounds that inhibit influenza virus NA enzymatic activity. The assays can be used in high throughput formats to screen large numbers of compounds found in diversified combinatorial libraries to identify candidate antiviral drugs or lead compounds, or to generate an activity profile that can be used as a fingerprint to detect influenza virus NA in clinical samples. The assay systems can be formatted in kits which can be used by the health practitioner at the point of care or the patient.

In one embodiment, the presence of influenza virus NA or the enzymatic activity of NA is used as a marker for the detection of influenza virus in a clinical sample. To this end, a detectable neuraminidase inhibitor (e.g., a labeled

neuraminidase inhibitor) can be contacted with the clinical sample -- binding of the detectable neuraminidase inhibitor to the sample indicates the presence of NA, and therefore, the influenza virus. Alternatively, the enzymatic activity of NA can be detected using a labeled substrate for NA which generates a detectable signal when enzymatically processed by neuraminidase. At least two approaches can be employed: the sample can be combined with a labeled substrate that is specific for the influenza virus NA -- generation of a detectable signal directly indicates the presence of influenza NA, and therefore, the influenza virus. Alternatively, a non-specific labeled substrate can be used in the presence and absence of a NA specific inhibitor -- attenuation of a detectable signal in this assay indirectly indicates the presence of influenza NA, and therefore, the influenza virus.

In accordance with the methods of the present invention, it is possible to evaluate the binding affinities of a library of diverse molecules for the thousands of potential binding sites present in a complex biological sample and generate a pattern of binding affinities exhibited by the sample which provide a unique fingerprint for that sample. In one embodiment of the present invention, it is possible to evaluate the binding affinities of a library of diverse molecules comprising specific and non-specific substrates and inhibitors of influenza neuraminidase which may be present in a biological sample and generate a pattern of binding affinities to identify a specific strain of influenza.

In another embodiment, the present invention relates to rapid, specific, high through-put screening assays to identify novel agents for their ability to interact with neuraminidase or some other viral component. In one embodiment, agents which interact with neuraminidase can be detected by combining influenza virus neuraminidase with a test agent and detecting the interaction of the test agent using the combinatorial screening methods of the present invention. In accordance with this embodiment, agents which

modulate influenza virus neuraminidase activity can be detected by combining a labeled specific or non-specific substrate of influenza virus neuraminidase with influenza virus neuraminidase and an agent. Those agents which attenuate the enzymatic processing of the substrate will be considered inhibitors of influenza virus neuraminidase activity.

The invention further encompasses the novel agents identified by the screening assays described herein. The invention relates to therapeutic modalities and pharmaceutical compositions for the treatment of viral infections using neuraminidase as the target for intervention. The present invention more particularly relates to therapeutic modalities and pharmaceutical compositions for the treatment of influenza virus infection by targeting neuraminidase. The present invention also relates to the use of antiviral agents identified by the present invention in combinatorial therapies with other known antiviral agents to inhibit viral replication.

The invention is based, in part, on the Applicants' design of sensitive, rapid, homogenous assay systems that permit detection of NA in samples, including but not limited to complex biological samples. The homogenous assay systems of the invention utilize robust detection systems that do not require separation steps for detection of NA. The preferred detection systems are fluorescence polarization and chemiluminescence.

The present invention is described in terms of neuraminidase by way of example and not by limitation, the combinatorial screening assays of the present invention may also be directed to detecting modulators or inhibitors of other viral proteins including, hemagglutinin, nuclear export protein, matrix proteins, nucleoprotein, and RNA directed RNA polymerase proteins in order to identify potential inhibitors of viral infection.

4. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to novel methods for detecting and diagnosing viral infections based on the combinatorial screening assays and detection methods of the invention which encompass contacting highly diversified libraries of compounds with biological samples which create fingerprints to allow for the identification of specific molecular differences existing between biological samples. The successful application of the combinatorial screening assay and detection method of the invention requires at least three components: (1) a diverse ligand library (probes); (2) a source of clinical samples (control and test samples); and (3) a sensitive assay for detecting ligand/receptor interactions. The combinatorial screening methods of the present invention may be designed as highly sensitive assays for diagnosis of viral infection. In another embodiment, the combinatorial screening assays of the present invention are used as sensitive high through-put screening tools to identify novel agents which interact with neuraminidase, and thus identify potential agents for the treatment of influenza viral infection.

The present invention relates to rapid, specific assay systems and kits for the diagnosis of influenza virus infection on the basis of the detection of influenza virus neuraminidase in clinical samples. In accordance with the present invention, a labeled substrate which binds specifically to influenza neuraminidase is combined with a clinical sample, and a detectable signal is generated by the enzymatic processing of the substrate by neuraminidase. This assay directly indicates the presence of influenza virus neuraminidase and therefore the virus. Alternatively, a labeled non-specific substrate of influenza neuraminidase is combined with a clinical sample, and a neuraminidase specific inhibitor.

The attenuation of a detectable signal in this assay indirectly indicates the presence of influenza virus

neuraminidase and therefore the virus. Another assay determines the presence of influenza virus in a clinical sample by detecting the interaction of a labeled neuraminidase specific inhibitor with influenza virus neuraminidase. In this assay a detectable signal will only be generated if influenza virus neuraminidase is present in the clinical sample.

The present invention further relates to rapid, specific, high through-put screening assays to identify novel agents such as drugs, ligands (natural or synthetic), ligand antagonists, peptides, small organic molecules and the like, for their ability to interact with neuraminidase or some other viral component. The assay systems described provide methods for the identification of agents that interact with influenza virus neuraminidase and agents that modulate influenza virus neuraminidase activity. In the assay systems of the present invention, a biological sample containing the test sample is contacted with a library of probes, comprising both known ligands, i.e., a diverse set of specific and non-specific substrates and inhibitors, and unknown ligands, i.e., test compounds, and comparing the binding activity of the test compounds to the known compounds. Assay systems which identify novel agents that modulate influenza virus neuraminidase activity involve screening for agents which prevent influenza virus neuraminidase from interacting with its substrate. In yet another embodiment of the present invention, the high-throughput combinatorial screening methods may be used to detect highly specific inhibitors of neuraminidase enzymatic activity. Those agents which attenuate a detectable signal by inhibiting the enzymatic processing of the substrate will be considered inhibitors of influenza virus neuraminidase activity and may be used in the treatment of influenza virus infection.

The invention encompasses pharmaceutical compositions containing the novel agents identified by the screening assays described herein. The invention relates to therapeutic modalities and pharmaceutical compositions for

the treatment of viral infections using neuraminidase or some other viral component as the target for intervention. The present invention also relates to the utilization of antiviral agents identified in the present screening assays in combination with other known antiviral agents which inhibit viral multiplication.

4.1 LIGANDS/PROBES

In accordance with one embodiment of the present invention, "fingerprints" are established when known biological samples, such as sputum, blood, sera, tissue samples, cells, viruses, microorganisms, or small organic molecules including RNA, DNA, peptides and proteins, are exposed to a battery of known reagents to generate a panel of values which reflect a pattern of binding interactions. In accordance with the neuraminidase-based assays of the present invention, the diverse ligand library may, for example, comprise: specific neuraminidase substrates, non-specific neuraminidase substrates, specific neuraminidase inhibitors, non-specific neuraminidase inhibitors, samples of neuraminidase isolated from various species of viruses and microorganisms, specific and non-specific antibodies to neuraminidase, or any variation of the above.

The ligands or probes of the present invention include any biological molecule, either natural or synthetic and may consist of nucleic acids, including DNA or RNA, small organic molecules, peptides, proteins, glycoproteins, polysaccharides, saccharides or inorganic molecules.

Neuraminidase substrates that can be used as probes in the assays include, but are not limited to, N-acetylneuraminic acid (NANA), and derivatives thereof such as 4,7-dialkoxy-N-acetyl neuraminic acid derivatives, including 4,7-dialkoxy Neu5Ac which is a specific substrate for influenza A and B neuraminidase, but does not interact with parainfluenza 1,2,3,4 virus, mumps, respiratory syncytial virus, adenovirus or bacterial neuraminidase, and 4-alkoxy-

Neu5Ac which is a non-specific substrate for influenza A and B neuraminidase, as described in U.S. Patent No. 5,719,020, incorporated herein by reference in its entirety; and chromagenic derivatives of NANA including a 4-position modified NANA as described in WO 91/09972 incorporated herein by reference in its entirety; 9-position modified NANA as described in WO 91/10744 incorporated herein by reference in its entirety; 5-position modified NANA as described in WO 91/09971 incorporated herein by reference in its entirety; and 7- or 8-position modified NANA WO 91/09945 incorporated herein by reference its entirety. Neuraminidase substrates to be used in accordance with the assays of the present invention include trisaccharide derivatives of NANA and fluorogenic derivatives of NANA such as 4-methylumbelliferyl-NANA as described in U.S. Patent No. 5,453,533, incorporated herein by reference in its entirety. The concentration of substrate used in the assays will be based on the results from titration experiments. The concentration(s) of substrate which results in the highest sensitivity for the detection of influenza virus neuraminidase activity will be used.

Known influenza virus NA specific and non-specific inhibitors can be used as probes in the assay system including but are not limited to, natural inhibitors, including *Staphylococcus aureus* glycoliporproteins which inhibit influenza A_0, A_1, A_2 neuraminidase as described in GB 2,238,049, incorporated herein by reference in its entirety; non-carbohydrate inhibitors, such as inhibitors of influenza A and B neuraminidase as described in U.S. Patent No. 5,453,533, incorporated herein by reference in its entirety; piperidine compounds which inhibit viral and bacterial neuraminidase as described in WO 98/17647, incorporated herein by reference in its entirety; aromatic compounds which inhibit viral and bacterial neuraminidase as described in US Patent No. 5,512,596, incorporated herein by reference in its entirety; carbocyclic based compounds, which inhibit viral

and bacterial neuraminidase as described in US 5,763,483, incorporated herein by reference in its entirety; 2-deoxy compounds, which have anti-orthomyxovirus and anti-paramyxovirus activity, as described in WO 92/06691, incorporated herein by reference in its entirety; 2-deoxy-2,3-didehydro-Na-cetylneuraminic acid derivatives and analogs, as described in WO 91/16320, incorporated herein by reference in its entirety; 6-carboxamidodihydropyran derivatives as described in WO 96/36628, incorporated herein by reference; and general class inhibitors such as piperidine compounds which inhibit both viral and bacterial neuraminidase, as described in WO 96/26933, incorporated herein by reference in its entirety. The assays make use of the fact that the specific nature of the binding of the inhibitors to influenza virus neuraminidase are known. The concentration of inhibitor used in the assays will be based on the results from titration experiments. The concentration(s) of inhibitor which results in the highest sensitivity for the detection of influenza virus neuraminidase will be used in the assay system. Furthermore, probes used in the assay system can consist of compounds that can be substrates or inhibitors, such as analogs of neuraminic acid having a 6-position spacer group, which have a detectable label or surface-binding partner at the end of the spacer for concentration on solid surface/detection as described in WO 97/32214, incorporated herein by reference in its entirety.

4.1.1 LABELING OF LIGANDS/PROBES

Described herein are methods for detectably labeling molecules capable of interacting with influenza virus neuraminidase. The neuraminidase-interacting molecules, neuraminidase substrates and inhibitors, that are used in the assay systems described above can be labeled, tagged, or conjugated such that a signal is generated when the molecule interacts with neuraminidase. The labels, tags, or

conjugates include, but are not limited to, fluorescent compounds, radioactive bases, and chemiluminescent compounds.

Probes/Ligands can be detectably conjugated to fluorescent compounds, including but not limited to, fluorescein (FL) 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid (BO or BODIPY), 4-methylumbelliferyl, fluorescein, isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluoroescamine. The interaction between neuraminidase and fluorescently labeled neuraminidase-interacting agents can be detected by a spectrofluorimeter or preferably, by analyzing the mixture by fluorescence polarization.

Substrates for neuraminidase can be also conjugated to chromagenic and fluorescent compounds, such as coumarin. When these compounds are conjugated to the substrates they are undetectable. The hydrolysis of the conjugated compound by the influenza virus neuraminidase results in the production of a visually detectable pigment or a fluorescent compound which is detectable by a spectrofluorimeter.

It is also possible to label a neuraminidase specific inhibitor with a radioisotope such as ^{32}P, ^{125}I, or ^{135}I. The interaction between neuraminidase and the neuraminidase specific inhibitor can be detected by such means as the use of a gamma counter or a scintillation counter or autoradiography.

In a preferred embodiment, a neuraminidase specific or non-specific substrate is conjugated to a chemiluminescent compound such as hydroxyphenyldioxetane. In this case the enzymatic processing of the substrate by neuraminidase will lead to the emission of a photon which can be detected by a photomultipler tube or a charge coupled device (CCD) camera.

The binding interaction between a probe and a component of a biological sample may also be detected by ELISA (enzyme linked immunosorbent assay). The probe can be labeled or conjugated to such molecules as biotin, streptavidin or digoxigenin. Probes labeled with these molecules can be

detected using enzyme conjugated antibodies specific for the label. Alternatively, the probe can be labeled with an antibody, which may or may not be conjugated to an enzyme. An antibody not conjugated to an enzyme can be detected by a secondary antibody that is conjugated to an enzyme. The enzyme conjugated antibody will react with an appropriate substrate in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorometric or by visual means. Enzymes which can be used to detectably label an antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase, and acetylcholinesterase.

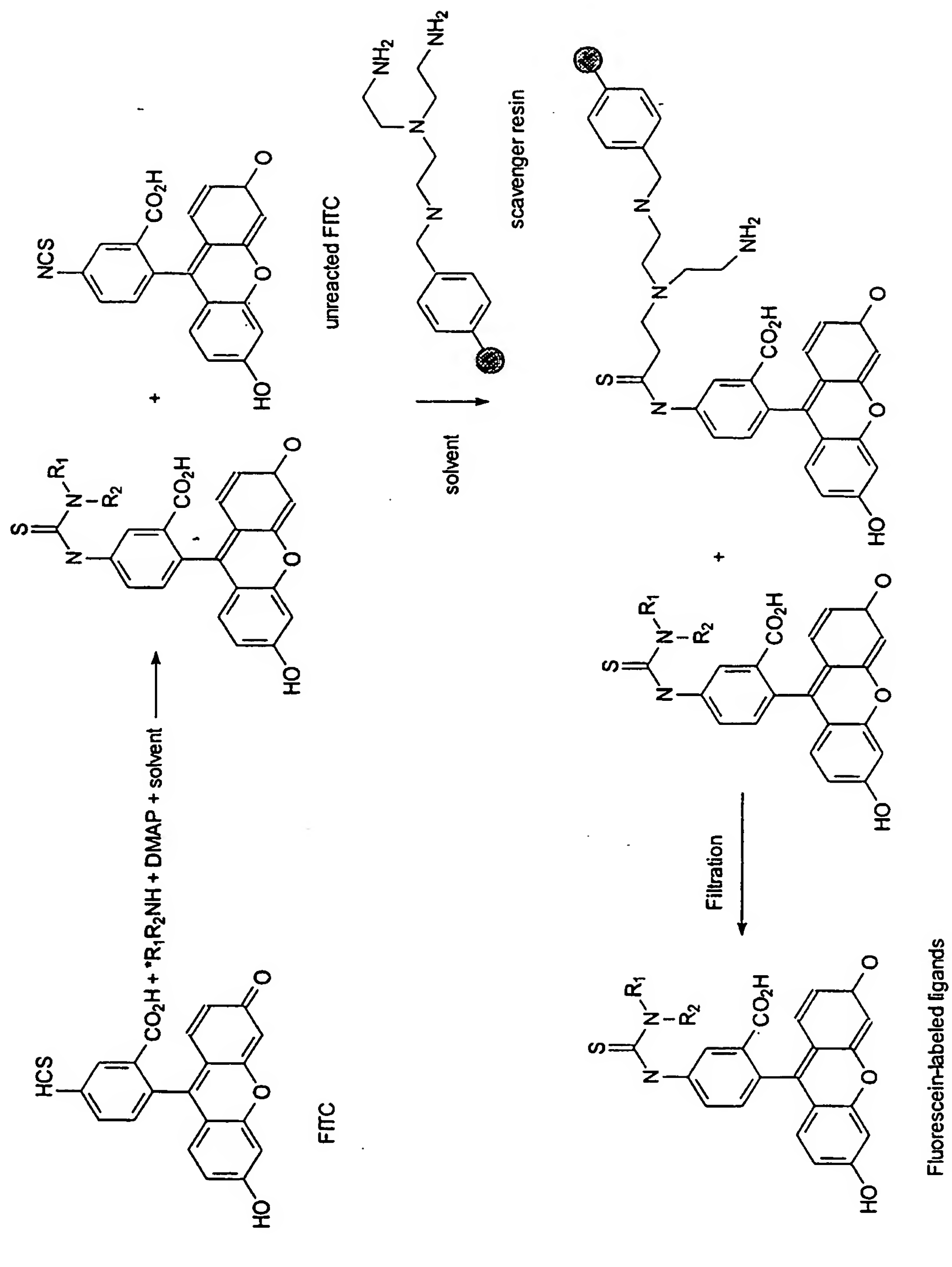
4.1.2 SYNTHESIS OF LIGAND LIBRARIES

The methods of the present invention may use ligand libraries synthesized according to any techniques known to those skilled in the art. Preferably, they are made using conventional solution phase reactions or solid phase synthetic techniques. Organic molecules of interests, such as biologically active compounds containing primary or secondary amine group, or hydroxyl groups, or thiol groups, or aldehydes or ketones, or carboxylic acids, can be labeled directly with suitable fluorescent molecules (dyes) in solution to give the corresponding fluorescent-labeled ligands. These methods and dyes are described in Haugland, R.P. Handbook of Fluorescent Probes and Research Chemicals, 6^{th} Ed., 1996. In a preferred embodiment of the invention, the solution phase syntheses are carried out in a suitable polar organic solvent or solvent mixture such as DMF, DMSO, THF using a slightly excess of dyes to ensure complete labeling. The resulting fluorescent-labeled ligands are

purified by standard techniques in organic synthesis such as liquid-liquid extractions using acid or base, crystallizations and chromatography (thin-layer or column). Alternative purification methods, such as liquid-solid phase extractions using polymer-bound scavengers to removal the unreacted dyes followed by simple filtrations can also be used as described in the following examples (*See*, Obrecht, D and Villalgordo, J.M., Solid-Supported Combinatorial and Parallel Synthesis of Small-Molecular-Weight Compound Libraries, Pergamon, 1998, Chapter 3.)

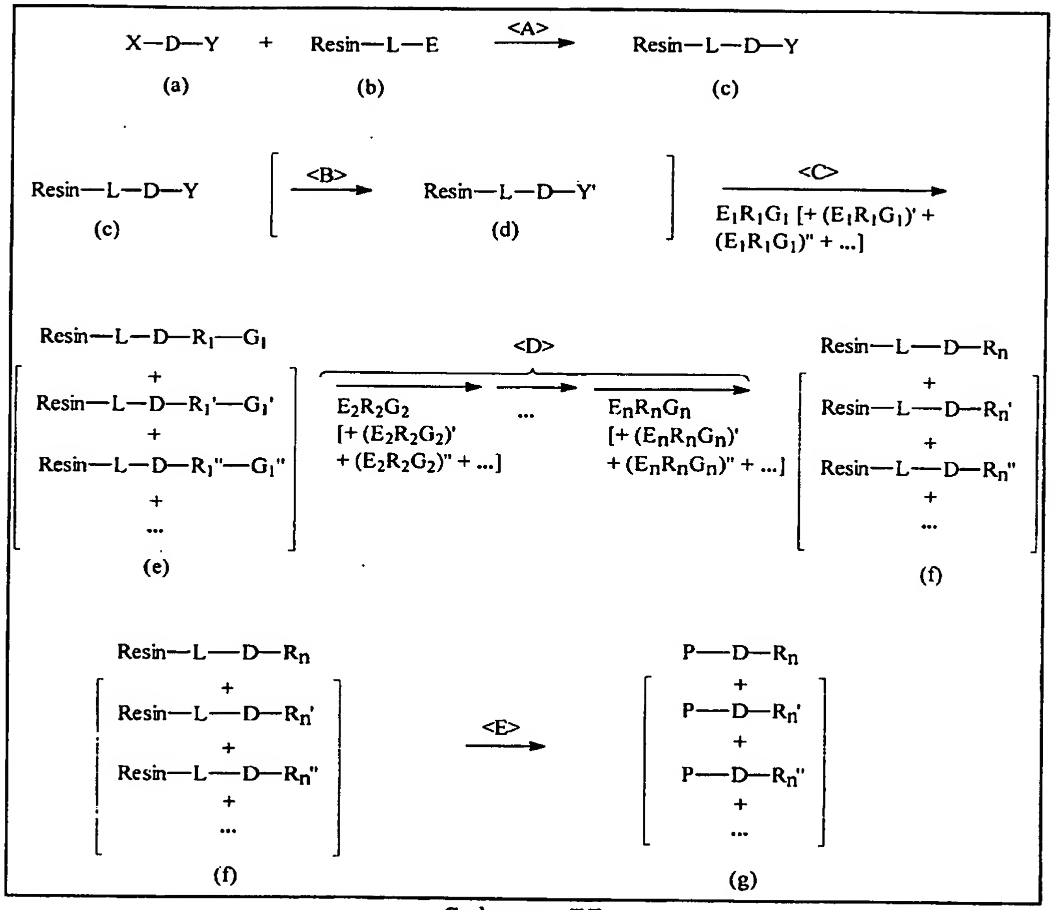
Scheme I. Solution phase reactions (products were purified by scavenger resin)

In one embodiment of the present invention, the libraries of this invention are made using conventional solid phase techniques. *See*, e.g., Bodanszky, *Principles of Peptide Synthesis* (Springer-Verlag: 1984); Bodanszky, *et al.*, *The Practice of Peptide Synthesis* (Springer-Verlag: 1984); Barany and Merrifield *The Peptides: Analysis, Synthesis and*


FITC
*R1R2NH + DMAP + solvent
unreacted FITC
scavenger resin
solvent
Filtration
Fluorescein-labeled ligands

Biology Vol. 2, Chapter 1 (Academic Press: 1980); Atherton, *et al.*, *Bioorg. Chem.* Vol. 8 (1979). This is because solid phase synthesis has several advantages over more traditional synthetic methodologies. For example, large excesses of reagents or starting materials can be used to drive individual reactions to completion, and purification and isolation can be performed by simple filtration and washing since the products are attached to solid supports. Furthermore, the relative site isolation of resin-bound species inhibits many types of intermolecular side reactions.

A solid phase synthetic method that has been found to be particularly suitable for the synthesis of the libraries of this invention is described below. Capable of quickly and efficiently forming diverse libraries of fluorescent-labeled ligands, this method comprises two general steps. In the first, a fluorescent dye is covalently attached to a solid support. In the second step, which may be repeated as many times as necessary, the immobilized dye is reacted with a compound or mixture of compounds to form the desired mixture of ligands. The present invention encompasses assays using libraries adhered to the solid supports upon which they were made, or adhered to different solid supports. It is preferred, however, that the mixture of ligands be cleaved from the support in a third step. This optional third step is included in the preferred embodiment of the synthetic method of this invention shown in Scheme II:



Scheme II

wherein <A>, <B>, <C>, <D> and <E> represent reaction conditions suitable for the formation of the desired products or intermediates represented by Formulas (b) - (g), and brackets (*i.e.*, [ ]) represent optional parallel or sequential reactions, reactants, and/or products.

According to Scheme II, a dye molecule of Formula (a) is selected:

X-D-Y

Formula (a)

wherein D is a fluorescent moiety, and X and Y are functional groups independently selected from the group consisting of halogens, alcohols, nitros, thiols, ethers, esters, carboxylic acids, α-halo carboxylic acid derivatives, amines, amides, and protected and unprotected derivatives thereof. Examples of dye molecules of Formula (a) include, but are not limited to: fluorescein derivatives such as dichlorotriazylaminofluorescein (DTAF), dichlorosulfofluorescein (DCSF), and nitrofluorescein; tryptophan derivatives; coumarin derivatives; napthyl derivatives; bipyridine (bpy) derivatives; tripyridine derivatives; cyanines; rhodamines and organometallic complexes such as $Ru(bpy)_3$ and derivatives thereof. The selection of dye molecules depends on a number of factors including, for example, size, solubility, immunity to degradation under solid phase reaction conditions, absorption and emission wavelengths, quantum yield, and quantum yield and emission wavelength sensitivity to the surrounding chemical environment. Many of these factors, and the synthesis of these and other suitable compounds, are readily determined from the literature. *See*, e.g., Haugland, R.P., *Handbook of Fluorescent Probes and Research Chemicals* (6^{th} ed.; 1996).

Also according to Scheme II, a reactive substrate of Formula (b) is selected:

Resin-L-E

Formula (b)

wherein Resin represents any solid support suitable for solid phase synthesis; L is a linker attached to the solid support; and E is a leaving group bound to L. Suitable solid supports include, for example, polystyrene-divinylbenzene (PS-DVB) copolymer and polyethylene glycol-PEG-PS-DVB copolymer. Wang (polymer-bound 4-benzyloxybenzyl alcohol) and Rink resins, with and without suitable linkers attached, are available

from Aldrich Chemical Co., Milwaukee, WI; Novabiochem, San Diego, CA; and Advanced Chemtech, Louisville, KY.

A linker L-E is selected so that its bond to the solid support is readily cleaved under the reaction conditions represented by <E> in Scheme II. Suitable linkers are known to those skilled in the art and include, for example, halogens, thiols, alcohols, ethers, esters, aldehydes, ketones, carboxylic acids, nitros, amines, amides, silanes, and protected and unprotected derivatives thereof. The attachment of such linkers to solid supports may be accomplished by methods well known to those skilled in the art. *See*, e.g., Bunin, B.A., *The Combinatorial Index*, Academic Press, 1998.

In addition to the criteria described above, the linker L-E is selected so that it will form a covalent bond with the fluorescent moiety D of the dye molecule of Formula (a) under reaction conditions <A> to yield an immobilized dye of Formula (c):

Resin-L-D-Y

Formula (c)

Suitable reaction conditions <A>, which depend upon Resin, L, E and X, are well known to, or easily determined by, those skilled in the art. Generally they include the use of a solvent that causes the resin to swell and react with X. Suitable solvents include, for example, dimethylformamide (DMF), 1-methyl-2-pyrrolidinone (NMP), tetrahydrofuran (THF), CH_2Cl_2, and mixtures thereof. The reaction conditions <A> also may include a base such as diisopropylethlamine (DIPEA), triethylamine, dimethylaminopyridine (DMAP), or N-methylmorphaline (NMM), to neutralize the acid generated during the reaction.

The immobilized dye of Formula (c) serves as a foundation upon which the ligands of the library (represented by Formula (g) in Scheme II) are formed. If the reactive

moiety Y is protected, however, it must be deprotected prior to additional reactions. This optional deprotection to form the deprotected moiety Y' is performed under reaction conditions represented by <B> in Scheme II. These conditions, which vary depending upon the protecting group, are well known to those skilled in the art. *See*, Greene, T.W. and Wuts, P.G.M., *Protective Groups in Organic Chemistry* (2nd ed.; 1991).

The immobilized dye is then reacted under reaction conditions <C> with a compound of formula $E_1R_1G_1$ to yield a compound of Formula (d):

$$\text{Resin-L-D-}R_1\text{-}G_1$$

Formula (d)

wherein E_1 and G_1 may be the same or different, E_1 is a leaving group or protecting group, G_1 is either the terminal end of R_1 or a leaving group or protecting group, and R_1 represents any chemical fragment which comprises at least one protected or unprotected reactive moiety that enables the addition of R_1 to the fluorescent moiety D under suitable catalytic and/or deprotecting conditions. Suitable reactive moieties include, but are not limited to, halogens, thiols, alcohols, ethers, esters, aldehydes, ketones, carboxylic acids, nitros, amines, amides, silanes, and protected and unprotected derivatives thereof. Suitable reaction conditions <C> include those which have been developed for solid phase combinatorial chemistry. *See*, e.g., Brown, R., *Contemporary Organic Synthesis*, 216 (1997); Felder, E.R., and Poppinger, D., *Adv. Drug Res.*, 30:111 (1997); Balkenhohl, F., *et al.*, *Angew. Chem. Int. Ed. Engl.* 35:2288 (1996); Hermkens, P.H.H., *et al.*, *Tetrahedron* 52:4527 (1996); Hermkens, P.H.H., *et al.*, Tetrahedron 53:5643 (1997); Thompson, L.A., *et al.*, *Chem. Rev.* 96:555 (1996); and *Chem. Rev.* 97(2) (1997).

Exemplary addition reactions include that of a primary amine with an aldehyde to form an imine, which in turn can react with a variety of different moieties including, for example, β-lactams, pyrrolidines, thiozolidinones, and amides. Acid groups are equally flexible, and be used, for example, with aldehydes, amines and isonitriles under Ugi multicomponent condensation conditions to form either small amides or heterocyclic compounds.

As indicted by Scheme II, the immobilized dye molecules of Formula (c) may also be reacted with a mixture of compounds, each of which is different but is of the general formula $E_1R_1G_1$; *i.e.*, $E_1R_1G_1 + (E_1R_1G_1)' + (E_1R_1G_1)'' + \cdots + (E_1R_1G_1)^i$, wherein i is the number of compounds in the mixture and is an integer preferably less than about 50. In such a case, a mixture of compounds of Formula (d) is produced, each possessing a different R_1G_1 fragment; *i.e.*, Resin-L-D-R_1G_1 + Resin-L-D-$(R_1G_1)'$ + Resin-L-D-$(R_1G_1)''$ + ⋯ + Resin-L-D-$(R_1G_1)^i$. It is preferred, however, that the compounds of Formula (c) only be reacted with one compound of formula $E_1R_1G_1$.

Because many pharmacologically active compounds contain reactive moieties such as amines and carboxylic acids, the present invention contemplates that such compounds are encompassed by the formula $E_1R_1G_1$, in which case further reaction may or may not be desired. If, however, the R_1 fragment of the compound(s) of Formula (d) is a reactive moiety, n-1 subsequent addition reactions may be performed under reaction conditions that are collectively referred to in Scheme II by <D>, wherein n represents the number of moieties bound to the fluorescent moiety D, and is an integer of preferably less than about 100.

As above, each of these subsequent addition reactions may employ both single compounds or mixtures of compounds of formulas $E_kR_kG_k$, wherein k is an integer between 2 and n-1, R_k is the k^{th} moeity bound to the immobilized fluorescent moiety D (via the k-1 moieties already bound to D), E_k and G_k are the same or different, E_k is a leaving group or protecting group,

G_k is the terminal end of R_k or a leaving group or protecting group, and R_k represents any chemical fragment which comprises at least one reactive moiety that enables the addition of R_k to the immobilized compound(s). Suitable reaction conditions <C> include the use of catalysts, deprotectants, and the like which facilitate the addition of R_k to the immobilized fluorescent compounds.

Completion of the reactions described above forms either an immobilized compound of Formula (f):

$$\text{Resin-L-D-}R_n$$

Formula (f)

or a mixture of immobilized compounds of Formula (f); *i.e.*, Resin-L-D-$(R_1R_2R_3\cdots R_n)$ + Resin-L-D-$(R_1R_2R_3\cdots R_n)'$ + Resin-L-D-$(R_1R_2R_3\cdots R_n)''$ + $\cdots$ + Resin-L-D-$(R_1R_2R_3\cdots R_n)^m$, wherein m has a maximum value of about i*n when i is equal to the number of compounds in the $E_kR_kG_k$ mixture having the largest number of compounds. For the sake of simplicity, G_n is omitted from Formula (f) because the terminal end of the ligand (*e.g.*, R_n) undergoes no further addition reactions.

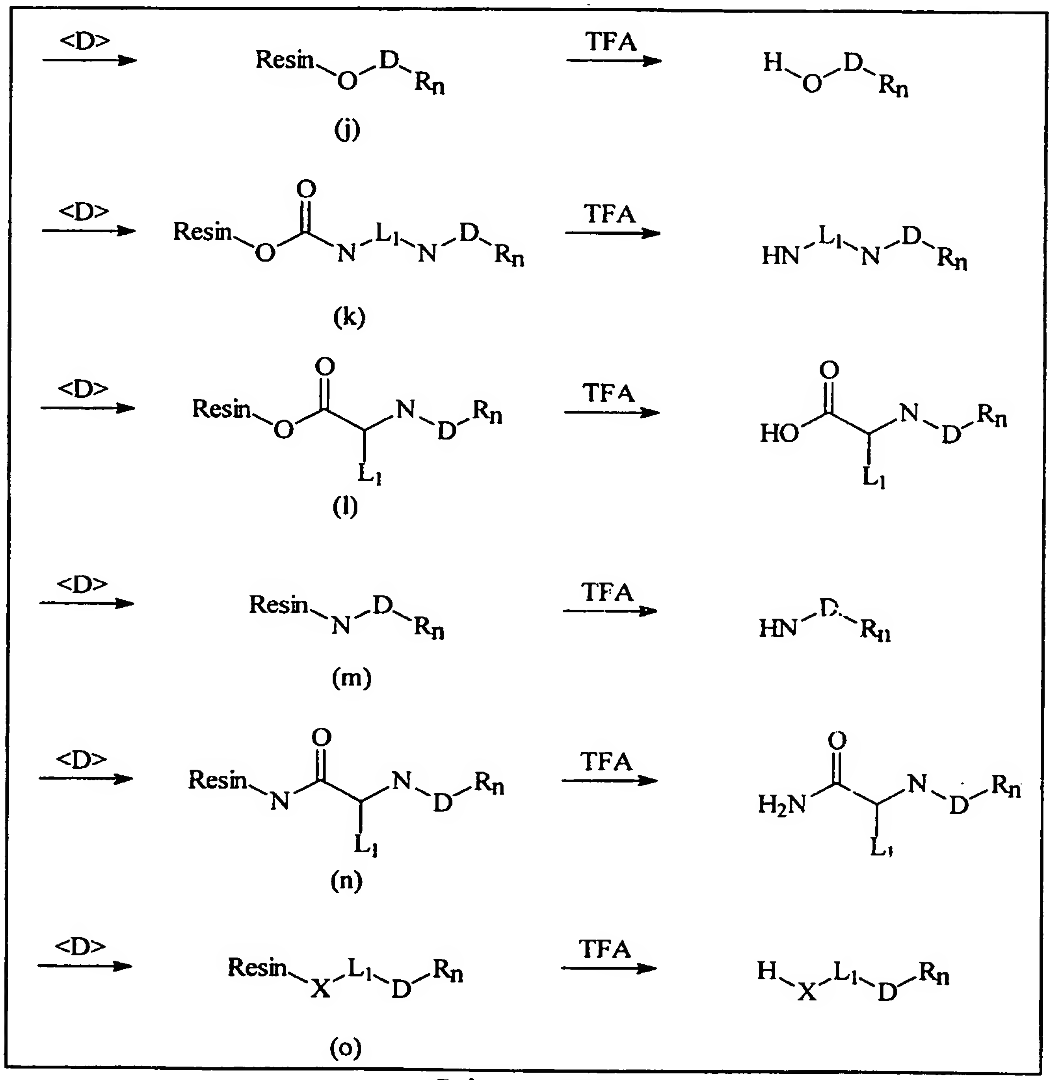
In the final step of Scheme II, the dye-ligand compounds are cleaved from the solid support under reaction conditions <E> to yield a library of compounds of Formula (g):

$$\text{P-D-}R_n$$

Formula (g)

wherein it is to be understood that Formula (g) encompasses all possible compounds and mixtures of compounds produced by the reactions indicated in Scheme II. Suitable cleavage conditions <E> are known to those skilled in the art, and depend upon the bond between Resin and L. Cleavage may be accomplished under acidic or basic conditions, or may be photoinduced. Many suitable cleavage methods have been reported in the literature. For example, some cleavage

reactions accomplished by treating the modified resin of Formula (f) with trifluoroacetic acid (TFA) in methylene chloride are shown in Scheme III:



Scheme III

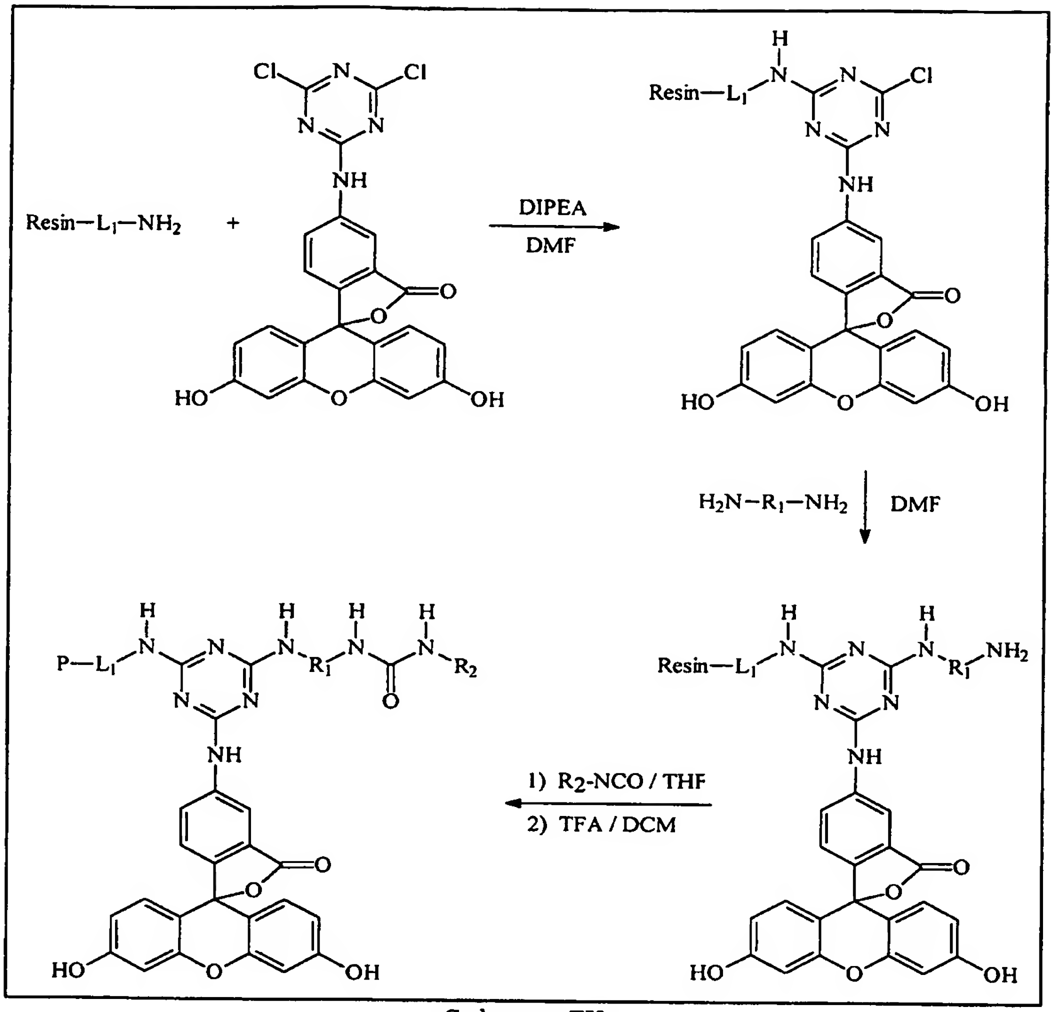
wherein (j) is a Wang resin derivative; (k) is a Wang carbamate resin derivative; (l) is a Wang amino acid resin derivative; (m) is a Rink resin derivative; (n) is a Rink

amino acid resin derivative; and (o) is a trityl or chlorotrityl amine (*i.e.*, X= NH) or alcohol (*i.e.*, X=O) resin derivative; and L_1 represents any side chain or spacer stable under solid phase reaction conditions. Examples of suitable side chains include, but are not limited to, substituted and unsubstituted alkyl, aryl and aralkyl groups.

After cleavage, the solvent is preferably removed to isolate the fluorescent library. The library may then be dissolved in a solvent such as dimethylsulfoxide (DMSO) suitable for use in the assays of this invention.

Specific embodiments of the method of Scheme II are shown in Schemes IV - VIII. For clarity, these schemes do not show the reaction and formation of mixtures. It is to be understood, however, that each of the individual reactions shown represents the possibility of numerous parallel reactions.

A particular simplified embodiment of the general approach of Scheme II is shown in Scheme IV:



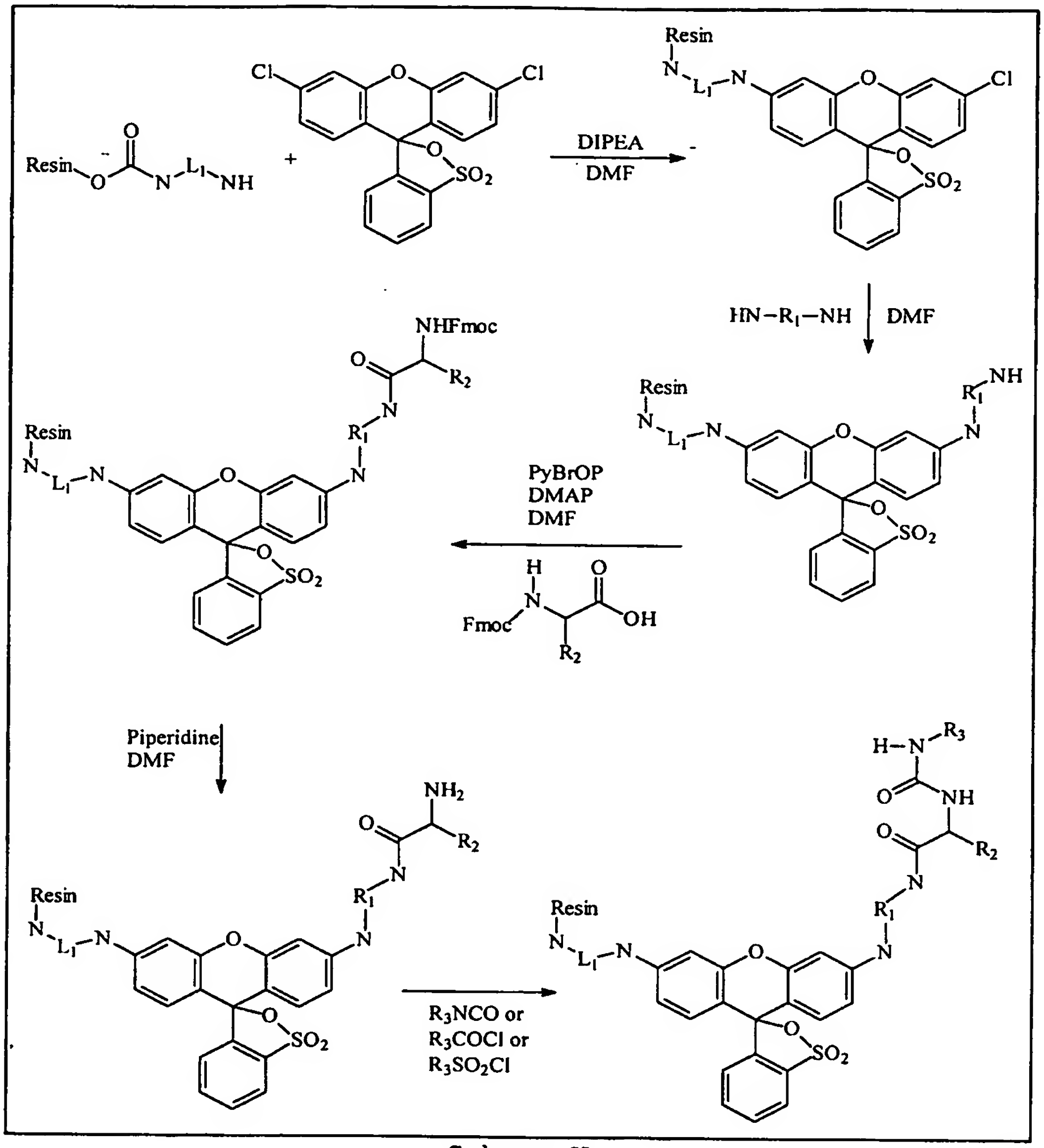
Scheme IV

wherein L_1 is any moiety that does not sterically hinder or otherwise inhibit the coupling reaction under the reactions conditions shown; P represents the end of L_1 after it has been cleaved from the solid support; and R_1 and R_2 are the same or different and may be any moieties desired to provide a library with preferred structural and reactive characteristics. Examples of suitable moieties include, but

are not limited to, substituted and unsubstituted alkyl, aryl and aralkyl.

According to Scheme IV, DTAF is immobilized upon a solid support using diamino carbamate Wang resin or amino acid Rink resin or diamino/amino alcohol trityl/chlorotrityl resin to give monochlorotriazylaminofluorescein resin. This reaction is preferably conducted at ambient temperature. DTAF is dissolved in a suitable solvent such as DMF, NMP, THF, methylene chloride, or mixtures thereof with between about 0.5 to about 3 equivalents of a base such as DIPEA, triethylamine, DMAP, or NMM. Substitution of the remaining chloride on the triazine ring with an excess of symmetrical diamine (preferably between about 2 to about 6 equivalents) in, for example, DMF or NMP at ambient temperature provides a new reactive group for further synthesis. This process is repeated as many times, and with as many different reactants, as desired, after which the resulting fluorescent compound or mixture of compounds is cleaved from the reactive support.

Another embodiment of the general approach of Scheme II is shown in Scheme V:

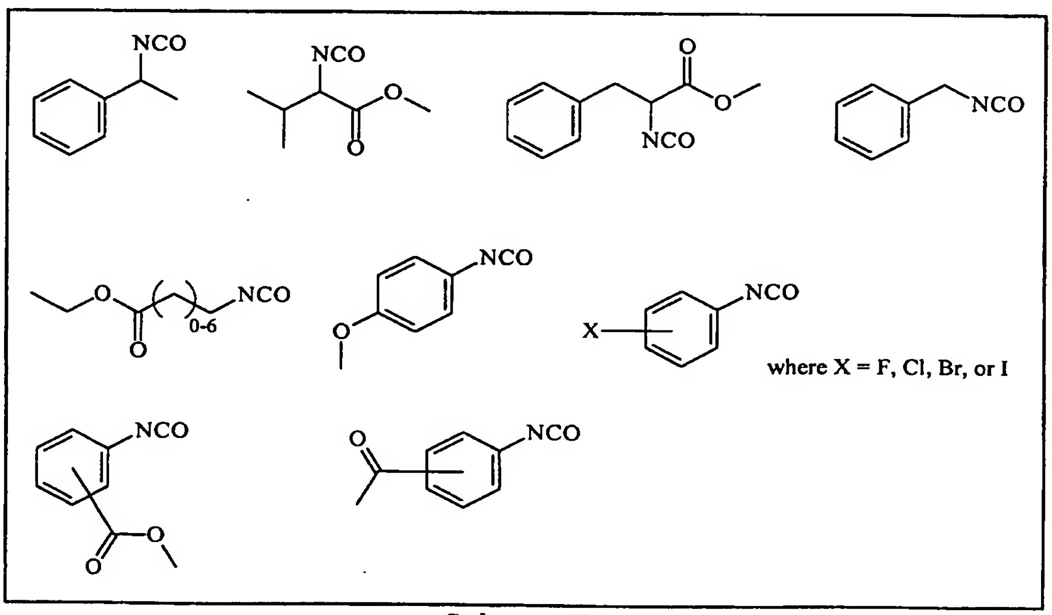


Scheme V

wherein DCSF is attached to a solid support via substitution of a chlorine atom by a secondary alkyl amine, and preferably a cyclic secondary amine, bound to a resin. L_1 thus forms part of a cyclic diamine. Suitable cyclic diamines include, for example, piperazine, homopiperazine, 4,4'-

trimethylenedipiperidine, and derivatives and isomers thereof. As shown, R_1 also forms part of a cyclic diamine, although HNR_1NH may be replaced by any compound having suitable reactive groups. R_2 and R_3 represent any moieties suitable for incorporation within the fluorescent ligands of the libraries of this invention and include, for example, the side chains of natural amino acids; substituted and unsubstituted alkyl, aryl and aralkyl; and the like.

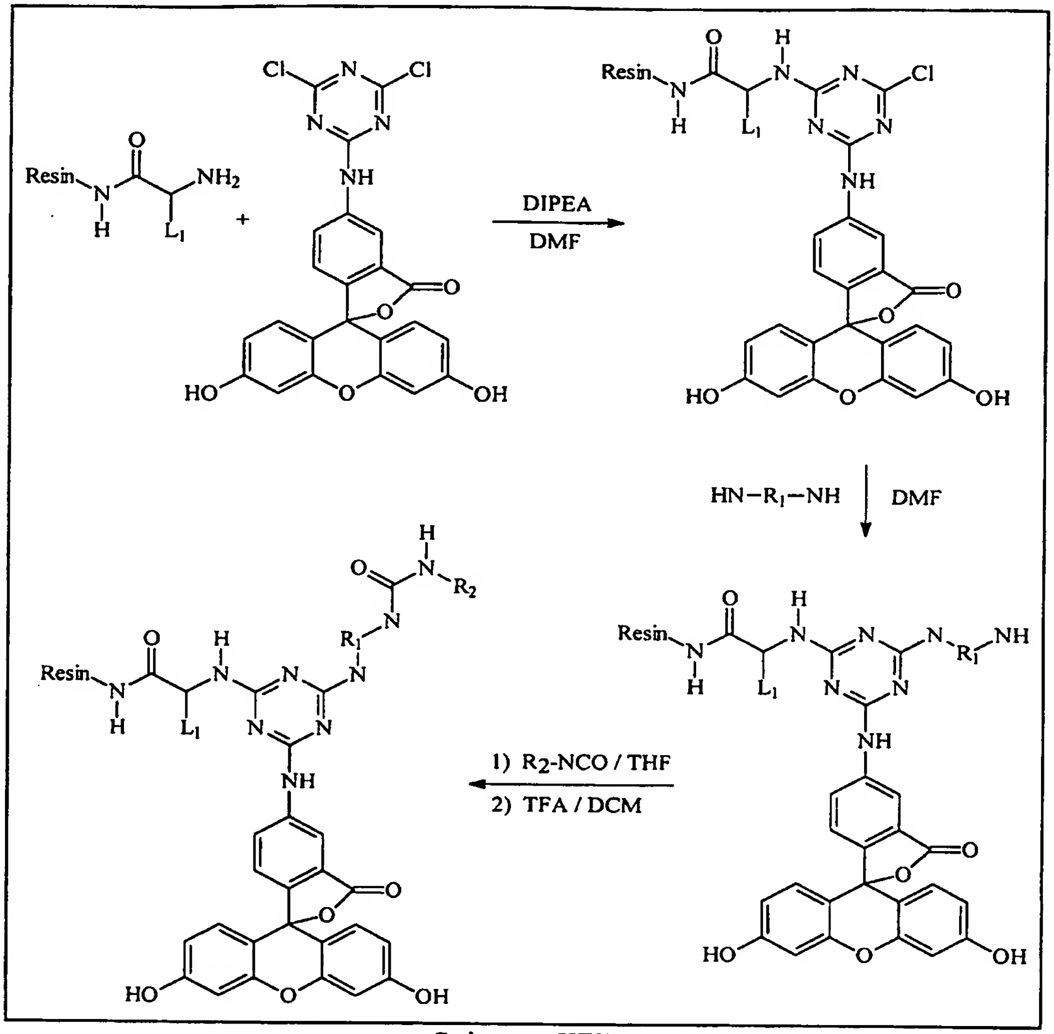
As shown in Scheme IV, an N-Fmoc protected amino acid is attached to the fluorescent resin by the reaction of the free amino group of the fluorescent compounds with the Fmoc amino acid under standard amide formation conditions (*i.e.*, PyBrOP/DMAP/DMF). After removal of the Fmoc group with piperidine in DMF, the new amino group provided by the amino acid can be derivatized with, for example, acid chlorides, chloroformates or isocyanates to give a variety of fluorescent labeled compounds. Non-limiting examples of suitable isocyanide compounds are provided in Scheme VI:



Scheme VI

As should be readily apparent to those skilled in the art, numerous other moieties (i.e., R_4, R_5, ···, R_n) comprising reactive moieties such as amino acids, acid chlorides, chloroformates, and isocyanates may be used to form the ligands bound to DCSF. Similarly, the chemical fragments to which the R_2 and R_3 groups of Scheme IV are attached may be replaced by any others which will allow the growth of the chain bound to the dye molecule provided that the reaction conditions are altered in a suitable manner.

A final embodiment of the general approach of Scheme II is shown in Scheme VII:



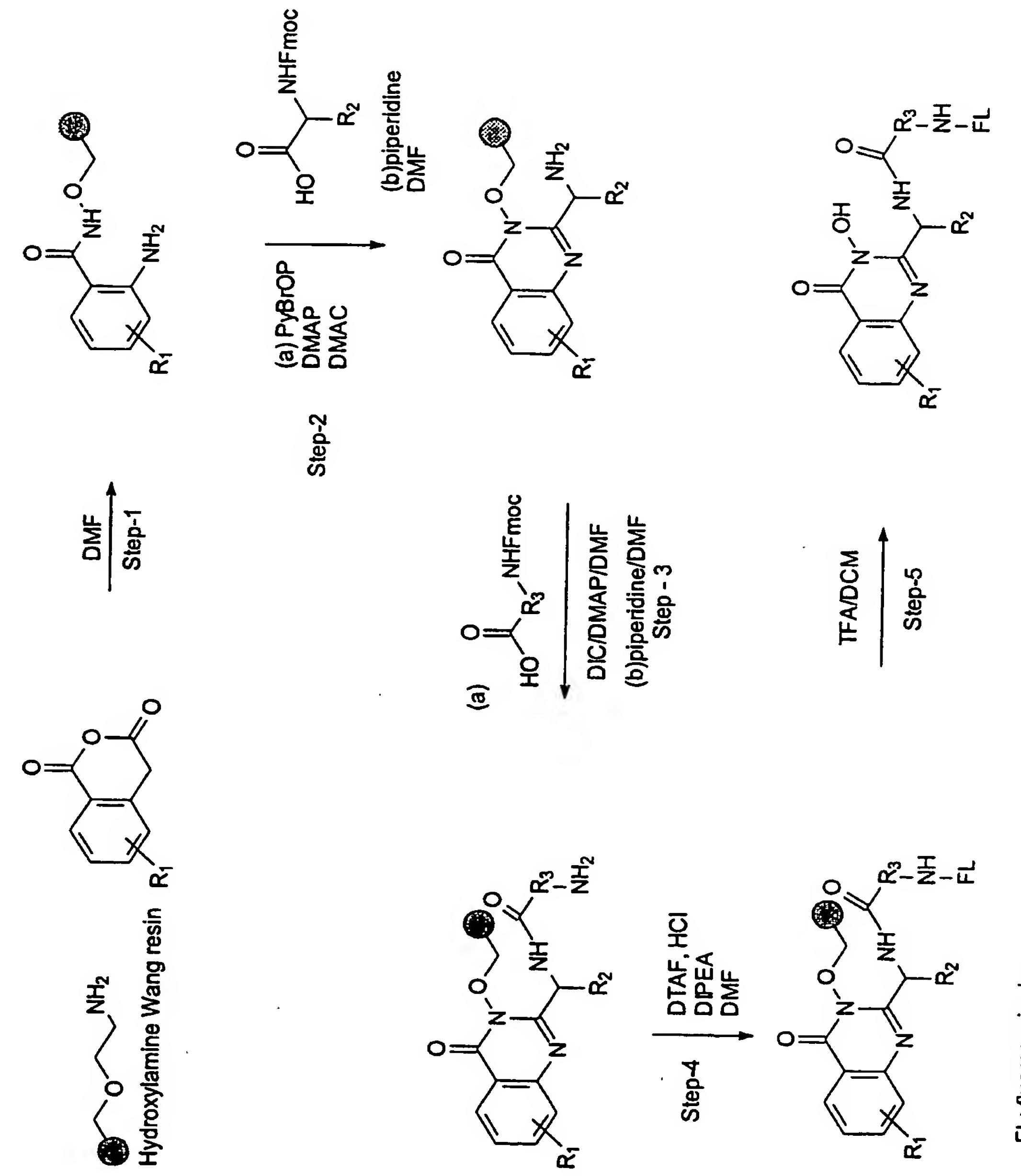
Scheme VII

wherein DTAF is bound to a Rink amino acid resin, and then subsequently derivatized by the methods described above. L_1, R_1 and R_2 are defined as above.

In addition to the above methods, fluorescent-labeled ligand libraries are also made by general solid-phase synthesis techniques (Obrecht, D. and Villalgordo, J.M., Solid-Supported Combinatorial and Parallel Synthesis of Small-Molecular-Weight Compound Libraries, Pergamon, 1998;

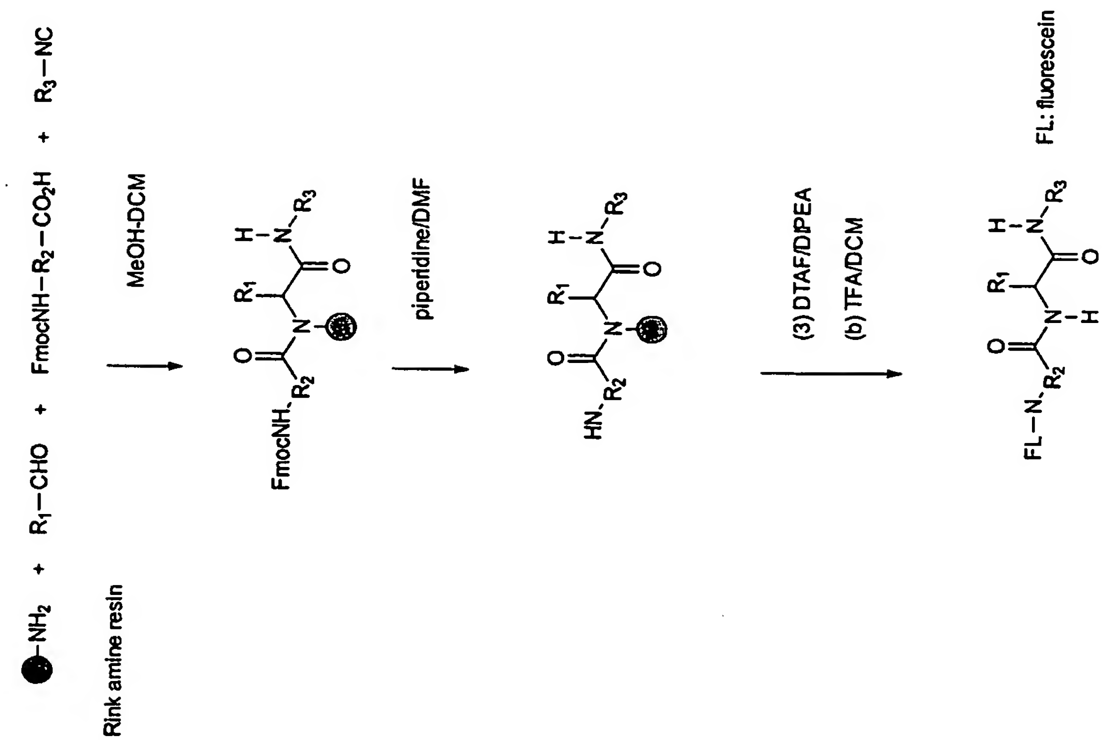
and Bunin, B.A., The Combinatorial Index, Academic Press, 1998). In a preferred embodiment of the present invention, the desired compounds are synthesized on the solid supports according the methods described in the literature. Before cleavage from the solid supports, the compounds on the solid supports are treated with suitable dyes to give the fluorescent-labeled ligands on resins. The ligands are then cleaved from the resins to give the fluorescent-labeled ligands.

In one approach, the ligands are synthesized in a linear fashion by reaction of a solid supported reactive block with different reactive blocks step by step. The dye is then added in the last step before cleavage to give the labeled ligands as shown in Scheme VIII. In this scheme, fluorescent-labeled N-hydroxyquinzolinones are prepared. Quinzalinones are one of the most common bioactive nitrogen containing heterocycles (*See*, Sinha, S. and Srivastava, M. in Progress in Drug Research, 1994, vol. 43, 143-238). They display a broad spectrum of biological and pharmacological activities in human and animals. They have been used as anticonvulsant, antibacterial and antidiabetic agents. Therefore, fluorescent-labeled quinzolines would be useful for diagnostic applications and for drug discovery.


Hydroxylamine Wang resin
DMF
Step-1
(a) PyBrOP
DMAP
DMAC
(b)piperidine
DMF
Step-2
(a)
DIC/DMAP/DMF
(b)piperidine/DMF
Step - 3
DTAF, HCl
DIPEA
DMF
Step-4
TFA/DCM
Step-5
FL: fluorescein dye

Scheme VIII

In another approach, the ligands are prepared in a convergent manner using multicomponent condensation reactions such as the Ugi condensation (Tempest, P.A., et al. *Angew. Chem. Int. Ed. Engl.* 1996, vol. 35, 640-642). Using this approach, the amine component is immobilized on the solid support as Rink amine resin and the aldehydes, Fmoc-protected amino acids, and isocyanides are added in excess to the resin swelled in mixture of MeOH/DCM (1:2 v/v) (Scheme IX). By combinatorial uses of different aldhydes, acids and isocyanides, a large number of ligands may be synthesized.


Rink amine resin
MeOH-DCM
piperidine/DMF
(3) DTAF/DIPEA
(b) TFA/DCM
FL: fluorescein

Scheme IX

4.2 BIOLOGICAL SAMPLES

The present invention provides methods for "fingerprinting" complex biological mixtures or samples containing viral neuraminidase which may be obtained from a wide variety of sources. Neuraminidase may be obtained from a patient sample, cells infected with a virus which expresses neuraminidase, recombinantly expressed neuraminidase or purified neuraminidase obtained by using standard molecular biology and protein purification techniques. The methods of the present invention may be utilized to identify specific phenotypical differences which exist between normal and abnormal, e.g., noninfected or infected cells and/or tissues. The methods of the present invention may also be applied to

identify or distinguish between species of microorganisms, viruses, bacteria, fungi or parasites.

The methods of the present invention detect all types of ligand/receptor interactions, whereby the neuraminidase receptor could be a purified protein, nucleic acids encoding NA or NA regulatory elements, or any molecules a shape that is capable of representing neuraminidase, i.e., interacting with neuraminidase specific probes or ligands. As used herein, the target receptor--neuraminidase may be referred to as-- "biological receptor", "receptor", "biological target", "target" and "component of a biological sample".

Accordingly, one aspect of the present invention is a method for characterizing an influenza viral infection, said method comprising identifying a pattern of binding interactions between neuraminidase receptors or targets present in biological samples and a library of ligands or probes, wherein the pattern of binding interactions provides a unique fingerprint for the pathology.

In accordance with the present invention, a "receptor" or "target" is a biological molecule which represents or mimics neuraminidase binding affinities or enzymatic activities including, but are not limited to, proteins, including enzymes, antigens, antibodies, lipids, nucleic acids including DNA and RNA, carbohydrates including lectins, cell surface proteins or receptors, etc.

The biological samples utilized in the method can be any sample that is a source of biological molecules, including but not limited to, biological materials such as blood sera, tissue samples, cell extracts, products from *in vitro* transcription and translation systems (obtained, for example by the method of U.S. Patent No. 5,654,150 issued to King *et al.*), and the like. Moreover, extracts derived from or fluids containing pathogenic organisms such as bacteria, yeast, fungi, viruses, protozoa, and the like may also be used. In these instances, ligands exhibiting high affinity and specificity for a protein or other receptor in the

pathogen may reveal new targets and can be tested for inhibitory effect against the pathogen.

The biological samples which may be screened in accordance with the methods of the present invention may be obtained from a wide variety of sources. By way of example, but not by limitation, biological samples or mixtures may be obtained from patients and include bodily fluids, blood, serum, mucous, including oral, rectal or intestinal mucosa, urine, feces, etc. In addition biological samples may include tissue samples, biopsy tissue, cell samples, including bone marrow cells, lymphocytes, immune cells, mucosal cells obtained from oral, rectal or intestinal mucosal linings, etc. In yet another embodiment, the biological samples or mixtures may encompass cell lysates or portions thereof, carbohydrates including lectins; proteins including glycoproteins, cell surface receptors, peptides; nucleic acids including DNA or RNA etc. In yet another embodiment the biological sample may be or may be derived from a virus, bacteria, microorganism or parasite or fluids containing such biological samples, e.g., testing water supplies for microorganism content.

The biological samples of the present invention may be obtained from individuals inflicted with a disease, disorder, or pathology infected with a virus, bacteria or other microorganism. In yet another embodiment, the biological samples may be generated by exposing a tissue, cells in culture, cell extracts etc. to a toxin or pathogenic agent, or by genetically engineering the genome of a cell in culture to encode a mutation or protein or peptide known to be associated with any given pathology or disorder.

Collections of biological materials as sources for clinical samples may be obtained from hospitals or national research facilities.

4.3 SCREENING ASSAYS TO DETECT INFLUENZA VIRUS NEURAMINIDASE IN CLINICAL SAMPLES

Recognizing that clinical materials are often available in only a limited supply for any given pathology, the third element of the invention, the sensitive assay systems, must be capable of detecting binding interactions occurring in only microliters of sample and in addition, must be capable of detecting binding interactions of less than optimal affinity. The assay systems of the present invention must also be capable of eliminating or greatly reducing nonspecific binding of ligands to receptors present in the sample. Recognizing these factors, the combinatorial assay systems and detection methods of the present invention improve upon existing systems such as fluorescence polarization, scintillation proximity assays (SPA), and enzyme-linked immunosorbent assays (ELISA).

The present invention provides rapid, specific assays for detecting and diagnosing influenza virus. More specifically the present invention provides methods for detecting influenza virus neuraminidase (NA).

There are at least three approaches which can be utilized to detect influenza virus infection on the basis of NA. One approach is to utilize a labeled specific substrate for influenza virus neuraminidase which would give rise to a detectable signal upon enzymatic processing. This approach provides a direct indication of the presence of the neuraminidase and therefore the virus. Another approach is to a utilize a labeled non-specific substrate for influenza virus NA which would give rise to a detectable signal upon enzymatic processing, except in the presence of a specific inhibitor of influenza virus NA. The signal can be attenuated in the presence of a specific inhibitor of influenza virus NA. This approach provides an indirect indication of the presence of the neuraminidase and therefore the virus. A third approach is to utilize a labeled specific inhibitor of viral NA which would give rise to a detectable

signal upon interaction with NA. This approach provides a direct indication of the presence of neuraminidase and therefore the virus.

4.3.1 DIAGNOSIS OF INFLUENZA VIRUS INFECTION

Individuals can be diagnosed with influenza infection by obtaining a nasal (or throat) swab or aspirate from them. This can then be mixed in a small volume of carrier fluid. In a preferred embodiment, the swab or aspirate can be diluted in approximately 0.5ml of carrier fluid. A labeled specific or non-specific substrate for NA can be added to or contained in the carrier fluid. The combination of a NA substrate and a clinical sample will give rise to a detectable signal upon enzymatic processing by influenza virus NA. To ensure that the enzymatic activity detected when using a non-specific substrate is due to influenza virus neuraminidase, a specific inhibitor of influenza virus neuraminidase should be added to the reaction mixture. The addition of a specific inhibitor of influenza virus neuraminidase to a mixture containing a non-specific substrate for influenza virus NA and a clinical sample will attenuate a detectable signal. These two approaches in the diagnosis of influenza virus infection take advantage of the enzymatic activity of influenza neuraminidase.

Another approach in the diagnosis of influenza virus infection is to obtain a nasal swab or aspirate from an individual and to mix it in carrier fluid with a labeled neuraminidase specific inhibitor. The combination of a neuraminidase specific inhibitor and a clinical sample will give rise to a detectable signal if neuraminidase is present in the clinical sample. Detection of a signal in this assay will indicate the presence influenza virus.

In a preferred embodiment, these assays can be performed in a physician's office to diagnosis influenza virus infection. The assay systems described will provide the physician with a rapid, specific, and accurate method of

diagnosing influenza virus infection. In another preferred embodiment, the method of detection of neuraminidase is sensitive enough to allow approximately 100 particles or less to be detected.

4.4 HIGH THROUGH-PUT SCREENING ASSAYS TO IDENTIFY NOVEL AGENTS WHICH MODULATE NEURAMINIDASE ACTIVITY

In another embodiment, novel agents that modulate neuraminidase activity can be identified by high through-put assay systems. These novel agents can include, but are not limited to, drugs, ligands (natural or synthetic), ligand antagonists, peptides, small organic molecules and the like. One approach used to identify novel agents that modulate neuraminidase activity consists of mixing an agent in carrier fluid containing influenza virus or influenza neuraminidase and a substrate for influenza NA. A detectable signal will be generated if an agent does not inhibit influenza virus NA activity. Whereas, agents that inhibit neuraminidase activity will be detected by their ability to attenuate the signal generated by the enzymatic processing of a non-specific or specific influenza virus NA substrate by influenza virus NA.

4.5 HIGH THROUGH-PUT SCREENING ASSAYS TO IDENTIFY NOVEL AGENTS WHICH INTERACT WITH NEURAMINIDASE

In another embodiment, novel agents that interact with neuraminidase can be identified by rapid, specific, high through-put assay systems. These novel agents can include, but are not limited to, drugs, ligands (natural or synthetic), ligand antagonists, peptides, small organic molecules and the like. One approach to identify novel agents that interact with influenza virus neuraminidase consists of labeling agents and screening for those agents that interact with neuraminidase. Another approach consists of labeling neuraminidase and screening for agents that interact with neuraminidase. A number of different assay

systems can be utilized to detect the interaction between an agent and neuraminidase including, but not limited to scintillation proximity assays (SPA), DNA obstruction assays, fluorescence polarization assays.

4.5.1 SCINTILLATION PROXIMITY ASSAY

In SPA assays neuraminidase or agents can be bound to a scintillant loaded bead. In standard a SPA assay, the neuraminidase can be tagged with a scintillant-loaded bead and screened against radiolabelled agents in solution. However, the reverse of this arrangement in which the agent is attached to the beads and the neuraminidase is radiolabelled is possible. Multiple agents can be synthesized on a bead. In such a system, beads loaded with scintillant and coated with an agent are immersed in a fluid phase containing radiolabelled neuraminidase. If the labeled neuraminidase has affinity for a tagged agent and the two become bound, the resulting proximity of the radiolabelled neuraminidase and the scintillant in the beads leads to activation of the scintillant and the emission of light. If the labeled influenza virus neuraminidase has little or no affinity for an agent, the radiolabel will not accumulate sufficiently close to the scintillant to allow for energy transfer following radioactive decay. Because SPA does not require a washing step, it allows for the detection of relatively low affinity agent/neuraminidase binding interactions. In a preferred embodiment, beads are blocked with blocking agents such as albumen, detergent or powdered milk to block sites responsible for non-specific adsorption.

A modification of the SPA assay can be used in competitive-type screening procedures where the neuraminidase is immobilized to a scintillant-loaded bead and then placed in a solution containing a radiolabelled agent which is known to interact with neuraminidase. Agents with unknown affinity for neuraminidase are then added to the mixture and any substrate that successfully competes with the known agent for

the immobilized receptor will attenuate the amount of light emitted. The use of SPA in a high through-put screen is described in Wang, P., *Target Identification, Assay Development and High Throughput Screening in Drug Discovery*, in Sino-American Pharmaceutical Professionals Association (SAPA), The 5th Regional Symposium on Drug Discovery and Development, 1997, Kenilworth, NJ.

4.5.2 DNA OBSTRUCTION ASSAYS

The principles of this system are based upon the presence or absence of restriction enzyme activity upon a DNA construct that has been synthesized to include a single restriction site and an agent-reporter system. When the construct is contacted with neuraminidase, agent/neuraminidase interactions will block the restriction enzyme's access to its restriction site and prevent hydrolysis of the construct at the site. Constructs that remain intact can be isolated and the agent/neuraminidase interaction identified.

The following is a description of a DNA restriction site assay system. A single-stranded DNA oligonucleotide containing biotin at the 5' end and digoxigenin at the 3' end is annealed to a complementary oligonucleotide such that the annealed double-stranded oligonucleotide contains a single centrally located restriction endonuclease site. The complementary oligonucleotide is modified to contain a linker having a terminal amino group. The location of the amino group, in this case between the 5th A and the 6th T from the 5' end, is such that it does not interfere with the activity of the restriction enzyme toward the double stranded oligonucleotide. The resulting construct, wherein the sequence $\begin{matrix} GGATCC \\ CCTAGG \end{matrix}$ (SEQ ID. No. ___) is a restriction site, is shown below:

```
              NH2
          5'  |                   3'
          ATATATGGATCCATATATAT
biotin - UTATATACCTAGGTATATATAU - digoxigenin
          3'                      5'
```

(SEQ ID. No. ___)

The amino group can be derivatized with agents of a diverse library to form the construct shown below:

```
              N - Agent
          5'  |                   3'
          ATATATGGATCCATATATAT
biotin - UTATATACCTAGGTATATATAU - digoxigenin
          3'                      5'
```

(SEQ ID. No. ___)

The derivatization of the amino group can be accomplished during synthesis of the single stranded oligomer while it is still attached to the CPG bead, and alkaline cleavage then used to release the oligomer from the bead where it can then be annealed to the complementary biotin-digoxigenin oligomer. As an alternative method of attaching the agents, derivatized bases may be incorporated during the synthesis of the complementary oligonucleotide.

When incubated with the restriction enzyme specific for the restriction site according to procedures well known in the art, the derivatized construct is hydrolyzed at the restriction site to provide two sections as shown below:

```
                 N - AGENT
                 |

             ATATATG               GATCCATATATAT
biotin - UTATATACCTAG +                GTATATATAT - digoxigenin
```

(SEQ ID. No. ___)

Reaction of the hydrolyzed mixture with a streptavidin or avidin coated surface results in the immobilization of the biotin labeled section and the digoxigenin labeled section is

eliminated by washing. Reaction of the immobilized mixture with anti-digoxigenin-peroxidaze antibody provides a negative result because the digoxigenin labeled section has been eliminated from the mixture by hydrolysis by the restriction enzyme and subsequent washing.

When the intact agent-derivatized construct is mixed with influenza virus neuraminidase, those agents with high affinity for neuraminidase will bind to it. After an incubation period, the reaction mixture is diluted with an appropriate buffer and treated with restriction enzyme and then incubated with a streptavidin coated surface to immobilize the biotin molecules. The interaction between an agent and neuraminidase will block the access of the restriction enzyme to its recognition site and prevent hydrolysis of the DNA scaffold.

```
                 N-AGENT-NEURAMINIDASE
             5'   |                    3'
             ATATATGGATCCATATATAT
  biotin - UTATATACCTAGGTATATATAU - digoxigenin
             3'                        5'
```

(SEQ ID. No. ___)

In the absence of an interaction between an agent and neuraminidase, the restriction enzyme will hydrolyze the DNA scaffold and free the digoxigenin-labeled portion of the scaffold. A standard enzyme-linked immunosorbent assay (ELISA) with anti-digoxigenin antibody can be used to detect the presence of digoxigenin on the streptavidin surface. A detectable signal indicates that the interaction between an agent and neuraminidase blocked the access of the restriction enzyme to its restriction site.

This assay can be modified in several ways. First, neuraminidase instead of agents may be attached to the DNA scaffold. Secondly, a deletion of a base can be inserted in one of the strands of the double-stranded DNA scaffold and an

endonuclease can be utilized instead of a restriction enzyme. Similar to the previous example, the interaction of an agent and neuraminidase will block an endonuclease (i.e., mung bean nuclease or SI nuclease) from accessing the gap and thus, will prevent the hydrolysis of the DNA scaffold. Another way the assay can be modified is to label the 3' end of the DNA scaffold with radioactive bases instead of digoxigenin. Furthermore, the DNA scaffold can be replaced with a backbone composed of peptides or peptoids or any polymer with a centrally located bond that can be cleaved by a particular enzyme or other mechanism wherein the cleaving can be blocked by the interaction of neuraminidase with an agent.

4.5.3 FLUORESCENCE POLARIZATION

Another assay system that can be used to identify agents with affinity for influenza virus neuraminidase is fluorescent polarization. A fluorescence polarization assay is designed to measure the binding of a fluorescent-labeled compound to an unlabeled biomolecule. A fluorescence polarization-based assay can utilize fluorescence labeled compounds up to a molecular weight of approximately 10,000 to detect interactions with the influenza virus neuraminidase. The type of fluorescent labeled compounds that can be used include, but are not limited to, small organic molecules, peptides, small proteins, nucleic acids, lipids, and polysaccharides. Fluorescent molecules when excited with plan polarized light will emit light in a fixed plane only if they do not rotate during the period between excitation and emission. The extent of depolarization of the emitted light will depend upon the amount of rotation of the molecules, which is dependent upon the size of the molecule. Small molecules rotate more than larger molecules between the time they are excited and the time they emit fluorescent light. The optimum conditions of this assay will exist when the labeled compound is much smaller than the unlabeled neuraminidase to which it binds. An unbound small

fluorescent-labeled compound rotates rapidly and the emitted light is depolarized. The interaction between influenza virus neuraminidase and a fluorescent-labeled compound will increase the effective size of the fluorescent-labeled compound and thus, decrease the rotation of the compound, which will result in the emitted light remaining polarized. The intensity of emitted, polarized light can be measured by inserting a moveable polarizing filter in front of the detector. The intensities are measured in planes 90° apart and are many times designated the horizontal and vertical intensities. In some instruments the excitation filter is moveable while the emission filter is fixed. In certain other machines the horizontal and vertical intensities are measured simultaneously via fiber optics. Three companies, Pan Vera, BMG Lab Technologies, and LJL Biosystems, market research grade fluorescence polarization instruments and Abott provides clinical laboratory instrumentation. The value of fluorescence polarization is determined by the following equation:

$$\text{polarization} = \frac{\text{intensity}_{\text{vertical}} - \text{intensity}_{\text{horizontal}}}{\text{intensity}_{\text{veritcal}} + \text{intensity}_{\text{horizontal}}}.$$

Fluorescence polarization values are most often divided by 1000 and expressed as millipolarization units (mP).

5. EXAMPLES

5.1 DIAGNOSIS OF INFLUENZA VIRUS INFECTION

5.1.1 NEURAMINIDASE ACTIVITY ASSAYS FOR DIAGNOSING INFLUENZA

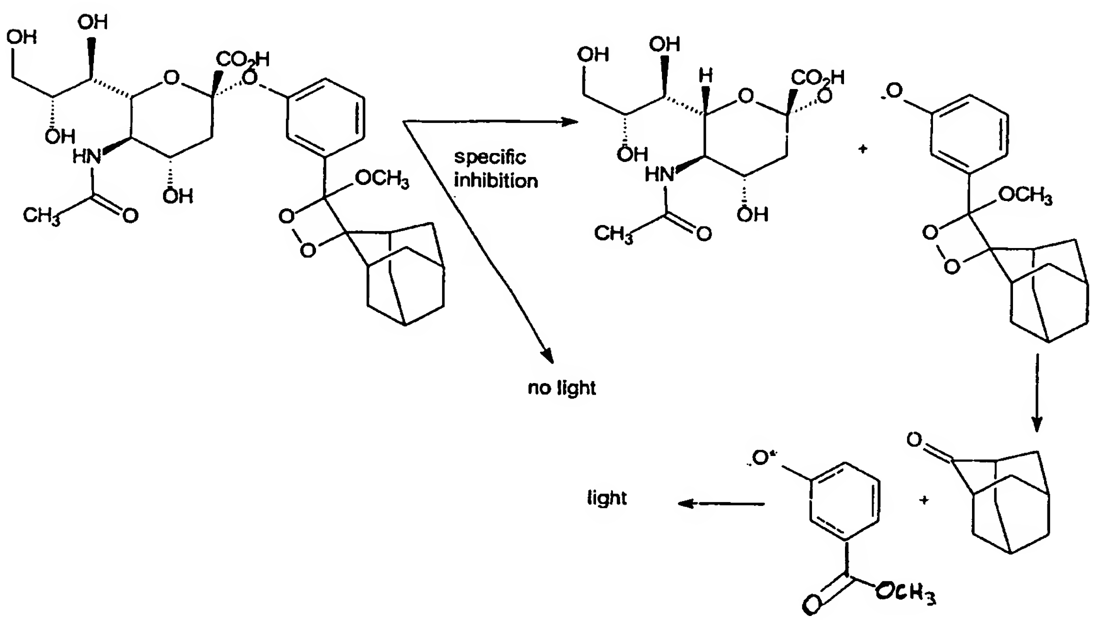
An individual suffering from influenza-like symptoms can be diagnosed in their physician's office. The physician swabs the individual's nasal passages and inoculates the swab in carrier solution containing N-acetylneuraminic acid, non-specific substrate for influenza virus neuraminidase. The

presence of neuraminidase in the clinical sample will result in the hydrolysis of the conjugated substrate. To ensure that the hydrolysis of the conjugated non-specific substrate was due to the presence of influenza virus, a specific inhibitor of influenza virus neuraminidase will be added to the mixture. The addition of a specific inhibitor of influenza virus neuraminidase, such as GR 217029 or GS4104, to the carrier solution containing the conjugated N-acetylneuraminic acid and the clinical sample will prevent neuraminidase from hydrolyzing of the substrate and no light will be detected. Thus, the specific presence of influenza virus in the clinical sample will be indicated by the attenuation of the detectable signal upon the addition of the specific neuraminidase inhibitor.

Alternatively, the presence of influenza virus neuraminidase in a clinical sample can be diagnosed by using a specific substrate of influenza virus neuraminidase. In this case, the glyceryl side chain of the N-acetylneuraminic acid can be altered to provide the specificity. The presence of influenza virus neuraminidase in the clinical sample will result in the hydrolysis of the specific substrate and a detectable signal will be generated.

A specific or non-specific substrate of neuraminidase can be conjugated to a chemiluminescent compound, such as hydroxyphenyldioxetane. Hydroxyphenyldioxetene conjugated N-acetylneuraminic acid in the presence of neuraminidase activity in the clinical sample will cleave the N-acetylneuraminic acid oxygen bond of the hydroxyphenyldioxetane, which destabilizes the dioxetane and leads to the emission of a photon (Scheme 10). The photons released can be detected by a photomultipler tube (a luminometer) or a charge coupled device (CCD) camera.
To ensure that the enzymatic activity detected is due to influenza virus neuraminidase, a specific inhibitor of influenza virus neuraminidase can be added to the reaction mixture. The addition of a specific inhibitor of influenza

virus neuraminidase will prevent the cleavage of the N-acetylneuraminic oxygen bond of the hydroxylphenyldioxetane and will attenuate the signal generated (Scheme X).



Scheme X

5.1.2 ASSAYS FOR DIAGNOSING INFLUENZA BASED UPON THE PRESENCE OF INFLUENZA NEURAMINIDASE

An individual suffering from influenza-like symptoms can be diagnosed in their physician's office. The physician swabs the individual's nasal passages and inoculates the swab in carrier solution containing a fluorescent labeled specific inhibitor, such as GR 217029 or GS4104. The sample is incubated with the labeled specific inhibitor for a predetermined period of time. The interaction between the inhibitor and influenza virus neuraminidase will affect the polarization of the light detected in the fluorescence polarization assay.

The value of the fluorescence polarization detected for the clinical sample will need to be determined in order to assess the significance of the polarization. The value of the polarization for the clinical sample will be compared to a positive control, consisting of influenza virus neuraminidase and the fluorescent labeled specific inhibitor, and a negative control, consisting of the coumarin labeled specific inhibitor. A fluorescence polarization value close to the positive control will indicate that the individual is infected with influenza virus.

5.2 HIGH THROUGH-PUT ASSAY FOR THE IDENTIFICATION OF NOVEL AGENTS

5.2.1 ASSAYS FOR THE IDENTIFICATION OF NOVEL AGENT THAT MODULATE INFLUENZA VIRUS NEURAMINIDASE

Novel agents that modulate influenza virus neuraminidase activity can be identified by combining agents with influenza virus neuraminidase and labeled substrates for neuraminidase. The substrates used may be non-specific (i.e., N-acetylneuraminic acid) or specific for influenza virus neuraminidase. The absence of an agent that modulates influenza virus neuraminidase will result in the hydrolysis of the conjugated substrate. Whereas, an agent that

modulates neuraminidase activity will prevent neuraminidase from hydrolyzing of the substrate and a detectable signal will be detected.

A specific or non-specific substrate of neuraminidase can be conjugated to a chemiluminescent compound, such as hydroxyphenyldioxetane. Hydroxyphenyldioxetane conjugated N-acetylneuraminic acid in the presence of influenza virus neuraminidase activity will cleave the N-acetyl neuraminic acid oxygen bond of the hydroxyphenyldioxetane, which destabilizes the dioxetane and leads to the emission of a photon. The photons released can be detected by a photomultipler tube (a luminometer) or a charge coupled device (CCD) camera. An agent that interacts with the active site of influenza virus neuraminidase or interacts non-competitively with influenza virus neuraminidase will prevent the cleavage of the N-acetylneuraminic acid oxygen bond of hydroxylphenyldioxetane and will result in the attenuation of the signal.

5.2.2 ASSAYS FOR THE IDENTIFICATION OF NOVEL AGENTS THAT INTERACT WITH INFLUENZA VIRUS NEURAMINIDASE

Novel agents that interact with influenza virus neuraminidase can be identified by combining fluorescently conjugated influenza virus neuraminidase with agents. The agents are incubated with the fluorescently labeled neuraminidase for a predetermined period of time. The interaction between the agents and influenza virus neuraminidase will affect the polarization of the light detected in the fluorescence polarization assay.

The value of the fluorescence polarization detected for the agent will need to be determined in order to assess the significance of the polarization. The value of the polarization for the agents will be compared to a positive control, consisting of fluorescently labeled influenza virus neuraminidase and a known agent that interacts with

neuraminidase, and a negative control, consisting of the fluorescently labeled influenza virus neuraminidase. A fluorescence polarization value close to the positive control will indicate that the agent interacts with influenza virus neuraminidase. This assay can also be performed with the agents conjugated to a fluorescent dye, as opposed to influenza virus neuraminidase conjugated to a fluorescent dye.

Other assay systems, such as a DNA scaffold obstruction assay, can be utilized to determine the interaction between influenza virus neuraminidase and an agent. Furthermore, in these assay systems the agents or influenza virus neuraminidase can be conjugated with a radioactive base or a chemiluminescent compound. The interaction between influenza virus neuraminidase and an agent can be detected by a gamma counter or scintillation counter when one or the other is conjugated to a radioactive base. Moreover, the interaction between an agent and neuraminidase can be detected with a luminometer when one or the other is labeled with a chemiluminescent compound.

The present invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the invention.

All references cited herein are incorporated herein by reference in the entirety for all purposes

What is claimed:

1. A method for detecting and/or diagnosing influenza virus infection, comprising:

(a) contacting a biological sample with a chemiluminescently labeled influenza virus neuraminidase specific substrate; and

(b) detecting enzymatic processing of the substrate by the generation of a chemiluminescent signal, in which the generation of the signal indicates the presence of influenza virus.

2. A method for detecting and/or diagnosing influenza virus infection, comprising:

(a) contacting a biological sample with a chemiluminescently labeled influenza virus neuraminidase non-specific substrate;

(b) adding to the reaction mixture an influenza virus neuraminidase specific inhibitor; and

(c) detecting enzymatic processing of the substrate by the generation of a chemiluminescent signal,

in which the attenuation of the signal indicates the presence of influenza virus.

3. A method for detecting and/or diagnosing influenza virus infection, comprising:

(a) contacting a biological sample with a chemiluminescently labeled influenza virus neuraminidase non-specific substrate, and a specific inhibitor of influenza virus neuraminidase; and

(b) detecting enzymatic processing of the substrate by the generation of a chemiluminescent signal,

in which the attenuation of the signal indicates the presence of influenza virus.

4. A method for detecting and/or diagnosing influenza virus infection, comprising:

(a) contacting a biological sample with a fluorescently labeled specific inhibitor of influenza virus neuraminidase; and

(b) detecting the presence of neuraminidase by the generation of a fluorescent signal,

in which the generation of the signal indicates the presence of influenza virus.

5. The method of Claim 4 in which the generation of the signal is detected by fluorescence polarization.

6. The method of Claim 1, 2, 3 or 4 in which the sample is obtained by swabbing or aspirating the nasal passage or throat of an individual.

7. The method of Claim 1, 2, 3 or 6 in which the appropriate positive and negative controls are utilized in the assays.

8. The method of Claim 1, 2 or 3 in which the substrate is conjugated to a precursor of a chemiluminescent compound.

9. The method of Claim 2, 3 or 4 in which the neuraminidase specific inhibitor is a drug, ligand (natural or synthetic), peptide, glycoprotein, protein, polysaccharide, saccharide, or inorganic molecule.

10. The method of Claim 1, 2, 3 or 4 in which the biological sample is a clinical sample obtained from a patient exhibiting symptoms of influenza virus infection

11. The method of Claim 4 wherein the fluorescent compound is 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-

indacene-3-propionic acid, fluorescein, isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde, fluoroescamine or coumarin.

12. A high through-put assay for identifying novel agents which modulate the activity of influenza virus neuraminidase, comprising:

(a) contacting a chemiluminescently labeled influenza virus neuraminidase substrate with influenza virus neuraminidase in the presence of a test agent; and

(b) detecting enzymatic processing of the substrate by the generation of a chemiluminescent signal,

in which the attenuation of the signal indicates that the agent is an inhibitor of influenza virus neuraminidase.

13. A high through-put assay for identifying agents which interact with influenza virus neuraminidase, comprising:

(a) contacting a chemiluminescently labeled test agent with influenza virus neuraminidase; and

(b) detecting the interaction of the neuraminidase and an agent by the generation of a chemiluminescent signal.

14. A high through-put assay for identifying agents which interact with influenza virus neuraminidase, comprising:

(a) contacting a fluorescently labeled test agent with influenza virus neuraminidase; and

(b) detecting the interaction of the neuraminidase and the agent by the generation of a detectable signal.

15. The assay of Claim 14 in which the generation of a signal is detected by fluorescence polarization.

16. The assay of Claim 12, 13 or 14 in which the test agent is labeled instead of the influenza virus neuraminidase.

17. The assay of Claim 12, 13, or 14 in which the agent is a drug, ligand (natural or synthetic), peptide, glycoprotein, protein, polysaccharide, saccharide, or inorganic molecule.

18. The assay of Claim 12, 13 or 14 in which the substrate is specific or non-specific for influenza virus neuraminidase.

19. The assay of Claim 12, 13 or 14 in which purified influenza virus or purified influenza virus neuraminidase are utilized.

20. The assay of Claim 12, 13 or 14 in which the assay system comprises:

(a) contacting a scaffold having a cleavable site with influenza virus neuraminidase, wherein the interaction between an agent and neuraminidase will block the cleavable site of the scaffold so that a detectable signal is generated; and

(b) adding the specific catalyst to the mixture, wherein the absence of an interaction will result in the cleavage of the scaffold so that a detectable signal will not be generated; and

(c) identifying an interaction between influenza virus neuraminidase and an agent based upon the generation of a detectable signal.

21. The assay of Claim 20 in which the scaffold is a double stranded DNA, a polypeptide or any polymer.

22. The assay of Claim 21 in which the cleavable site in the DNA scaffold is recognized by an endonuclease or a restriction enzyme site.

23. A kit for detecting and/or diagnosing the presence of influenza neuraminidase in a sample comprising:

(a) a chemiluminescently labeled neuraminidase substrate or inhibitor; and

(b) a means for detecting binding of the neuraminidase to the substrate in the sample.

24. The kit of Claim 23 wherein the substrate is specific for neuraminidase.

25. The kit of Claim 24 wherein the inhibitor is specific for neuraminidase.

26. A pharmaceutical composition, comprising an agent identified by assays described in Claim 12, 13 or 14 that inhibits influenza virus neuraminidase activity, in a physiologically acceptable carrier.

27. A pharmaceutical composition, comprising an agent identified by assay described in Claim 12, 13, or 14 that interacts influenza virus neuraminidase activity, in a physiologically acceptable carrier.

28. The pharmaceutical composition of Claim 26 in which the agent is a drug, ligand (natural or synthetic), peptide, glycoprotein, protein, polysaccharide, saccharide, or inorganic molecule.

29. The pharmaceutical composition of Claim 27 administered for the treatment of disease resulting from influenza virus infection.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/26945

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/70; G01N 33/53
US CL :435/5, 7.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5, 7.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, CAPLUS, WPIDS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,663,055 A (TURNER et al) 02 September 1997, see example 8 and claims.	1-29
Y	US 5,252,458 A (LIAV et al.) 12 October 1993, see claims 1-13.	1
Y	WO 97/32214 A1 (SCIENTIFIC MANAGEMENT PTY, LTD.) (BIOTA) 04 September 1997, see page 6, and claims.	1-29
A	LAMBRE, C.R. et al. Fluorometric Assay for the Measurement of Viral Neuraminidase in Influenza Vaccines. Vaccine. April, 1989, Vol 7, pages 104-105.	1-29

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"B" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 16 MARCH 1999	Date of mailing of the international search report 02 APR 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MARY K ZEMAN Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/26945

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YOLKEN, R.H. et al. Fluorometric Assay for Measurement of Viral Neuraminidase- Application to the Rapid Detection of Influenza Virus in Nasal Wash Specimens. The Journal of Infectious Diseases. October 1980, Vol. 143, No. 4, pages 516-523.	1-29

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/26945

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 7
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.